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# PRECIPITIN-RING TEST APPLIED TO FUNGI. II[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 445

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## I. Review of literature

This paper is a report on investigations, begun in 1927, to determine whether the precipitin test is serviceable for identification and classification of certain phytopathogenic Ascomycetes and Fungi Imperfecti and some of their non-pathogenic taxonomic allies. Fractions extractable with 0.85% NaCl solution from hyphae and spores of these fungi were used to produce immune sera in rabbits and for preparation of test antigens.

Following the attempts of SCHÜTZE (15) to apply the precipitin test to identification and classification of yeasts used in the brewing industries, and of CITRON (1) to use it for identification and detection of fungi pathogenic to man, MAGNUS and FRIEDENTHAL (7) attempted to determine its adaptability for phylogenetic studies of fungi. In the course of extensive use of the precipitin test by the Königsberg school in phylogenetic studies of the entire plant kingdom, STEINECKE (14) applied the test to representatives of the algae and fungi, and NEUHOFF and ZIEGENSPECK (13) used it to study the relationships of Basidiomycetes and representatives of other classes

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of fungi. The conclusions drawn from these studies were summarized by MEZ (**9, 10**) and MEZ and ZIEGENSPECK (**11**). While the work of this school incited numerous investigations bearing upon the suitability of the precipitin test for phylogenetic and biochemical studies of higher plants, serological studies of the phytopathogenic fungi have received little attention. Literature pertaining to these studies of higher plants has been recently summarized by SILBERSCHMIDT (**16**) and hence need not be discussed here.

Recently several papers bearing on the application of the precipitin test to phytopathogenic fungi have appeared. MATSUMOTO (**8**) applied serological tests, among them the precipitin test, to *Aspergillus* species, and concluded that the method has possibilities. COONS and STRONG (**2**) attempted to apply the precipitin test to *Fusarium* species. They were unable to produce sera with sufficiently high titers for precipitin tests, and so abandoned it in favor of the complement-fixation test. NELSON (**12**) reported limited success with the precipitin test using purified globulins of *Fusarium lini*. Like all others who have used fungal thalli as antigen sources, he had difficulty in obtaining saline extracts sufficiently potent to give good titers with antisera. LIM and KUROTCHKIN (**6**), and KESTON et al. (**3**), working with fungi pathogenic to man, used as test antigens water-soluble polysaccharide fractions and reported very high titers.

## II. Fungi used in test

To determine the efficacy of the precipitin test in differentiating the Fungi Imperfecti, representatives were chosen from the form genus *Fusarium*, which is taxonomically one of the most confusing genera of the Fungi Imperfecti, and phytopathogenically the most important division of the Phragmosporae. Identification and classification of the organisms considered species of this genus are often difficult or impossible, because when grown on artificial media they may fail to produce their distinguishing structures. Because of their biological and economic importance, the members of this genus seemed desirable material with which to begin a study of the antigenic and haptenic properties of phytopathogenic fungi.

In his latest contribution to the taxonomy of *Fusarium*, WOLLENWEBER (**17**) recognized 64 species with 79 varieties and 38 forms,

which he has arranged in 16 sections with 9 subsections, 12 of the sections including members for which the ascomycete stage has been established. This form genus is of additional interest because it includes many representatives which, although part of the etiological complex in diverse hosts or of distinct pathic events in the same host, are morphologically and physiologically differentiable only as varieties or forms.

The work was begun with three species of *Fusarium*, *F. conglutinans*, *F. cubense*, and *F. lycopersici*. It finally included the species and varieties listed in table I. The members of this list were added as the investigation progressed; consequently reciprocal tests have not been run with all of them. This list includes: (a) representatives which are known to be the conidial stages of different genera and species of the ascomycete order Hypocreales; (b) parasitic and non-parasitic representatives of the sections recognized by WOLLENWEBER (**17**); (c) pathogenic representatives which are characterized by host and even by symptom specificities, but which are not always readily differentiated morphologically.

The test was also applied to organisms so similar to *Fusarium* species that they had once been considered such. Hence *Cylindrocarpon album* (Sacc.) Wr. and a species of *Ramularia* were tested against one another and some of the *Fusarium* species. It was desirable to apply the test to members of the order Hypocreales which do not possess *Fusarium*-like conidial stages. Consequently *Neurospora tetrasperma* (Shear and B. O. Dodge) with monilioid conidia (*Monilia tetrasperma* Shear and Dodge) was used in tests with the conidial stage of *Gibberella saubinetii* (one way) and with other representatives with fusiform conidial fructifications.

Further experiments on relationship were made by testing members of the Hypocreales bearing fusiform or monilioid conidia, respectively, against members of the Pezizales with monilioid conidia or without macrospores, respectively. Consequently *Sclerotinia fructicola* (Wint.) Rehm, incitant of brown rot of stone fruits, which has a monilia stage, and *S. sclerotiorum* (Lib.) Mass., which bears no macroconidia but monilioid microconidia, were tested against the conidial stage of *N. tetrasperma* and several other representatives of the Hypocreales and of the genus *Fusarium*. Strains of *S. fructicola*

TABLE I

| Name used in this paper | Name assigned by Wollenweber (17) | Incitant of | Section | Conidial stage of |
|---|---|---|---|---|
| F. acuminatum Ell. et Ev. | F. scirpi Lamb et Fautr. var. acuminatum (Ell. & Ev.) Wr. | Carnation wilt | X Gibbosum | ..... |
| F. anthophilum Wr. | F. arcuatum Berk. & Curt. var. majus Wr. | " " | VIII Roseum | ..... |
| F. argillaceum (Fr.) Sacc. | No change | ..... | XVI Ventricosum | Hypomyces solani Rke. et Berth. |
| F. cepae (Hanz) emend. Lk. and Bailey | F. oxysporum (Schl.) Wr. | Onion bulb rot | XIV Elegans; subsect. oxysporum | ..... |
| F. conglutinans Wr. | No change | Cabbage yellows | XIV Elegans; subsect. orthoceras | ..... |
| F. conglutinans Wr. var. callistephi Beach | No change | China aster wilt | XIV Elegans; subsect. orthoceras | ..... |
| F. cubense E.F.S. | F. oxysporum Schl. forma 3 Wr. | Banana wilt | XIV Elegans; subsect. oxysporum | ..... |
| F. decemcellulare Brick | No change | ..... | III Spicarioides | Calonectria rigidiuscula (Berk. et Brme.) Sacc. |
| F. dimerum Penzig | No change | ..... | I Eupionnotes | ..... |
| F. fructigenum Fr. | F. lateritium Nees var. fructigenum (Fr.) Wr. | Decay of stem and fruits, especially of trees | IX Lateritium | Gibb. baccatae (Wallr.) Sacc. var. evonymi (Fuckel) Wr. |
| F. graminearum Schwabe* | No change | Wheat seedling blight | XI Discolor; subsect. saubinetii | Gibb. saubinetii (Mont.) Sacc. |
| F. javanicum Koorders | No change | ..... | XV Martiella | ..... |
| F. lycopersici (Sacc.) Wr. | F. bulbigenum Cke et Mass. forma 1 Wr. | Tomato blight | XIV Elegans; subsect. constrictum | ..... |
| F. moniliforme Sheld. | No change | Corn rot | XIII Liseola | Gibb. moniliformis (Sheld.) Wineland |
| F. nivale (Fr.) Cesate | No change | Snow mold of cereals | VI Arachnites | Calonectria graminicola (Berk. et Brme.) Wr. |

| | | | | |
|---|---|---|---|---|
| F. ossicolum (Berk et Curt) Sacc. | F. equiseti (Cda) Sacc. forma 1 Wr. | ..... | VI Gibbosum | ..... |
| F. oxysporum Schlechtendahl. | No change | Potato wilt | XIV Elegans; subsect. oxysporum | ..... |
| F. sambucinum Fuckel | No change | ..... | XI Discolor; subsect. saubinetii | Gibb. pulicaris (Fr.) Sacc. |
| F. semitectum Berk. et Rav. | No change | ..... | IX Arthrosporiella | ..... |
| F. sporotrichioides Sherb. | No change | Pea wilt | VII Sporotrichiella | ..... |
| F. theobromae App. et Strk.† | F. javanicum Koord. var. theobromae (App. et Strk.) Wr. | Cacao pod rot | XV Martiella | Hypomyces ipomoeae (Hals.) Wr. |
| F. trichothecioides Wr. | No change | Potato tuber dry rot | XI Discolor; subsect. trichothecioides | ..... |

* Two cultures of *F. graminearum* were used. One, derived from an ascospore of *Gibb. saubinetii*, is designated as *Gibb. saubinetii* #259; the other, from an ascospore of an English strain of *Gibb. saubinetii*, is designated *Gibb. saubinetii* English strain.

† Two cultures of *F. theobromae* were used. One, derived from an ascospore of *Hypomyces ipomoeae*, is designated *H. ipomoeae;* the other, derived from a conidial culture of *F. theobromae*, is designated *F. theobromae.*

(two monoconidial cultures which had appeared sufficiently diverse in several years of culture to be considered separate entities and were identified by Dr. JOHN W. ROBERTS as *S. fructicola*) and *S. sclerotiorum* were tested against each other. Finally, plus and minus strains of *N. tetrasperma* were used for a study of genotypically distinct strains of the same species.

*Sclerotinia sclerotiorum* was cultured in its vegetative and sclerotial stage and is designated *S. sclerotiorum* in this paper. *S. fructicola* was cultured in the conidial monilia stage, the two strains being designated *S. fructicola* no. 1 and no. 2 respectively. *Neurospora tetrasperma* was cultured in the conidial stage, *Monilia tetrasperma* Shear and Dodge. The sexually distinct strains are designated *Neurospora tetrasperma* A and B respectively, and the mixed culture as *N. tetrasperma* A+B.[2]

## III. Methods

A detailed report will not be given of every attempt made to devise a standard procedure, but only of those methods finally adopted.

CULTURE MEDIUM.—A search was made for a suitable protein-free liquid medium for *F. conglutinans*, *F. lycopersici*, and *F. cubense*. It was found that these organisms would grow in sufficient abundance in Richard's solution.[3] Cultures were grown in 100 cc. of solution in 250 cc. Erlenmeyer flasks because the yield per 1000 cc. of medium distributed in these units seemed to be greater than when larger units in larger flasks were used. Culture flasks were inoculated from a seeding flask (inoculated 4–5 days previously) containing a cloud of spores and hyphal fragments well dispersed by shaking. During the first days after inoculation the culture flasks were shaken to distribute the inoculum and then left undisturbed to facilitate mat production. Usually good mats developed in subdued light at 20°–

[2] All of these cultures were obtained from Dr. ALICE A. BAILEY except *Gibb. saubinetii*, obtained from Miss HELEN JOHANN; *F. nivale* obtained from Dr. A. S. DAHL; several strains of *F. cubense* obtained from Dr. J. R. JOHNSTON; *Cylindrocarpon album* and a *Ramularia* species obtained from Dr. C. D. SHERBAKOFF; *Neurospora tetrasperma* conidial strains A and B obtained from Dr. B. O. DODGE; and *Sclerotinia sclerotiorum* obtained from Dr. H. H. WHETZEL.

[3] 33.3 gm. sucrose, 6.6 gm. $KNO_3$, 3.3 gm. $KH_2PO_4$, and 1.7 gm. $MgSO_4$ in 1000 cc. distilled water.

30° C. within 3–4 weeks. All fungi used were grown in this medium. Some species grew more vigorously than others, so the yield of material (dry weight) per flask ranged from approximately 20 mg. to approximately 80 mg. If cultures were allowed to grow too long (2 months), the medium discolored, crystals formed which might become enmeshed in the mycelia, and black patches appeared on the lower side of the mats.

RECOVERY OF FUNGUS MATS.—Contents of the flask were poured upon filter paper (Schleicher and Schüll no. 595) on a Büchner funnel attached to a filter flask, and the medium drained off. The fungus mat was rapidly rinsed with three changes of distilled water and permitted to become semi-dry, at which time it could readily be peeled off the paper with forceps. Since extensive use was to be made of the fungus material for many months, it was necessary to grow large quantities and to dry the material preliminary to storage in tightly stoppered bottles. Various methods of drying with minimum opportunity for autolysis were tried. A drying apparatus was finally used which drew air at a temperature of 25°–30° C. over the damp fungus material loosely stacked in petri dishes. Mats were dried in 1–4 hours, depending on their thickness. The dried mats were stored in desiccators over sulphuric acid preliminary to pulverization.

PULVERIZATION OF MATS.—The mats were ground entirely in agate mortars, at first by hand fine enough to pass a 20-mesh sieve, then in an electrical grinding machine until sufficiently fine to pass a 100-mesh sieve. All attempts to grind the powder fine enough for suspensions usable in intravenous injections failed. The particles always swelled sufficiently to bring about the death of the animal. Powder prepared as just described when used in suspensions was suitable for intraperitoneal injection, such a suspension readily passing a 20-gauge needle.

EXTRACTION OF POWDERS.—The aim was to obtain clear extracts with sufficient protein content to make tests possible, yet free from lipoids which might interfere with the clarity of the test antigen and specificity of the precipitin test.

Preliminary trials on lipoid extraction involved using alcohol alone, in various concentrations, or using alcohol preceding or following ether extraction in the cold or in the reflux condenser. Petrol

ether (ligroin) (B.P. range 30°–60° C. max. B.P. 80°) was found to be the best lipoid solvent for these organisms. To minimize possible chemical change and loss of proteins, no extractions of the powders with alcohol and/or ether were made at temperatures above 25° C. For this same reason treatment of the material with selenium oxychloride as used by COONS and STRONG (2) was not adopted.

The following method of lipoid extraction was employed. Petrol ether was added to the desired amount of powder, shaken occasionally, and allowed to act on it for 1–15 hours at 25° C. After centrifugation this ether was decanted and fresh ether added. Usually three or four changes sufficed to give a fat-free test when a drop of ether was evaporated on a watch crystal. The ether was then decanted and the powder thoroughly dried preliminary to saline extraction. Using this method, powders extracted with 0.85% NaCl solution gave biuret tests, whereas some of the non-lipoid extracted powders did not. It was also found that in some cases (*F. cubense*, *F. lycopersici*, *F. conglutinans*) this treatment tended to enhance the antigenic properties of the injected material and the reactive properties of the test antigens. However, many of the fungus powders (*F. javanicum*, *F. fructigenum*) gave clear test antigens without lipoid extractions.

Grinding of fresh or dry fungus mats by hand or in a ball mill with various neutral or alkaline extractives was tried. This was abandoned because antigens sufficiently clear for intravenous injection or use as test antigens were not obtainable from the ground material, despite filtration and centrifugation. When only this material was injected intraperitoneally, it did not produce adequate titers for tests.

As suggested by MEZ (9), fungus powders were ground with pulverized pumice. A weighed amount of the powder was ground by hand with an equal volume of ether-extracted pulverized pumice preliminary to extraction with petrol ether and with salt solution. Since in some test antigens this procedure slightly increased the potency of the reaction, it was incorporated into the standard procedure.

Saline extractions of both inject and test antigen were tried for various periods of time at different temperatures to ascertain optimum conditions.

Following is a synopsis of the standard preparation of the antigens. For the test antigen, 1:50 stock dilution, 0.3 gm. of powder was ground with pumice (3 minutes), extracted with petrol ether until fat-free, dried, and extracted in 15 cc. of 0.85% saline solution for 18 hours at 0° C. After centrifugation, sparkling clear extracts were obtained. A few organisms used, such as the *Sclerotinia* species, required a longer centrifugation at higher speed. Extracts of several powders in concentrations greater than 1:50 (1:10 or 1:20) were readily made by this method. To prepare a clear 1:20 extract of *S. sclerotiorum*, however, filtration through hard filter paper (Whatman's no. 50) caked with fuller's earth (Hyflo) was required after centrifuging. For the injection antigens, powders were extracted directly in saline without preliminary pumice and petrol ether treatment. All antigens were stored at 0° C. Fresh lots of test antigens were usually prepared every 2 weeks. When stored a month or more they became slightly more acid but gave apparently valid reactions.

IMMUNIZATION.—Attempts to produce titers by intravenous injection of either clear extracts or suspended fungal material failed, the former because the titers were too low; the latter because the animals quickly succumbed to embolism. Neither did intraperitoneal injections alone prove adequate to incite potent antisera. A combination of intravenous injection of a clear saline extract of the powder and of intraperitoneal injection of the extracted powder resuspended in saline was found to give adequate titers.

The following standard procedure was used. For each series of injections per animal, 0.3 gm. of powder was extracted in 10 cc. of saline at 0° C. for approximately 16 hours, then separated by centrifugation to obtain a clear supernatant liquid. This liquid (8 cc. approximately) was used for intravenous injection; the residue, resuspended in 10 cc. of saline, was used for intraperitoneal injections (12 cc. approximately). Animals were bled for normal serum and then injected on three consecutive days according to the following schedule:

| | INTRAVENOUS INJECTION | INTRAPERITONEAL INJECTION |
|---|---|---|
| 1st day | 2 cc. | 3 cc. |
| 2nd day | 3 cc. | 4 cc. |
| 3rd day | Remainder (3 cc.+) | Remainder (4 cc.+) |

After a 5-day interval of rest this procedure was repeated. When the desired number of series of injections had been given, the animal was taken off feed the fourth day and bled the fifth day after the last injection was received. Two such series of injections usually produced antisera with adequate titers, some as high as 1:25,600; but in most cases approximately 1:3200. (An antiserum produced by this schedule is designated $I_2$ throughout this paper.) Forty cc. of blood furnished sufficient serum for tests against 20 to 25 antigens, allowing an adequate reserve for supplementary tests. No preservatives were added to the antisera, which were obtained aseptically and stored at 0° C. with relatively slight loss by spoilage.

Hot weather, moulting, feeding animals shortly before bleeding, all seemed to be factors which might contribute to opalescent serum. Such correlations were not consistent, however; the antisera of some animals never appeared opalescent, while those of others were frequently so.

PRECIPITIN TEST.—Numerous preliminary trials were made determining the most effective use of antisera and of test antigens to obtain maximum titers.

Tests were run with graded antigen dilutions against a constant serum (undiluted and diluted 1:1) and with graded serum dilutions against a constant antigen dilution. A 0.85% NaCl solution was used exclusively for the antigen dilutions; 10% glucose, glycerin, 1.7 and 2.55% NaCl solutions were used in serum dilutions. Tests with glucose-diluted serum gave sharp interphases but slightly lower titers; glycerin-diluted serum showed very sharp interphases but the titers were considerably diminished. Serum diluted with hypertonic saline showed very slight, if any, lowering in intensity of reaction. Serially diluted serum run against a constant antigen dilution could not be used because the density of the higher dilutions was such that clear interphases were not obtained.

Since sufficient serum could be obtained from each animal for tests with 20–24 test antigens, it was used undiluted against serial dilutions of antigens. Test antigen dilutions were prepared with 0.85% NaCl solution from a 1:50 stock extract of each fungus powder, to yield graded dilutions of 1:100, 1:200, 1:400, 1:800, to 1:6400, and when necessary, up to 1:25,600.

Approximately 0.05 cc. of each serum was distributed with a capillary pipette into each tube of a series of pyrex precipitin tubes (5 mm. diameter). The serum in each tube was then layered with approximately 0.05 cc. of the appropriate antigen dilution, separate pipettes being used for each dilution. The tubes were incubated at room temperature (28°–32° C.) and read at 1 and 2-hour intervals after layering. The symbols ±, 1, 2, 3, and 4 were used to represent the intensity of reaction as measured by the thickness and density of the layer of precipitate (ring) formed at the interphase; ± being the faintest discernible line, 4 the broadest, densest ring obtained, the other symbols representing intermediate values.

Preliminary studies showed that rings may appear at any time from less than one minute to 30 minutes after contact of antiserum and test antigen. Most of the second hour readings varied only slightly from those of the first hour, the usual difference consisting of a slight additional thickening of the rings to the next higher value (that is, a 2 ring became a 3 ring), or in the appearance of the last detectable reaction (±) in the next higher dilution (that is, a titer of 1:3200 of the first hour reading might rise to 1:6400 by the close of the second hour). Third and fourth hour readings usually were unsuccessful because extensive diffusion of the two fluids rendered the rings indefinite and their evaluation inaccurate. Attempts to estimate total precipitate by readings made after shaking the tubes (following the second hour reading) and holding them at 0° C. overnight proved unsatisfactory because of the difficulty in estimating the small amount of precipitate present, owing to the extreme curvature of the bottom of the tube.

Although extensive absorption tests were run, no one technique has yet been found which will give consistent results with all sera or with different samples of the same serum. The absorbing agents were the powders added directly to the sera, or extracts of powders, 1:10 in the *Fusarium* species and 1:20 in the *Sclerotinia* species studied. Powders were not extracted with petrol ether except in preparation of the extracts of the *Sclerotinia* species. Most rapid and complete absorption was obtained by using two volumes of extract to one volume of serum, mixing the two fluids thoroughly, and then incubating the tubes in a water bath (40° C.). After a suitable period

of incubation the tubes were transferred to 0° C., stored over-night, and centrifuged until clear the following day. Tests were run immediately with the decanted supernatant liquid against the standard series of test antigen dilutions. When required, test antigen dilutions under 1:50 were prepared from the extract used as the absorbing agent.

When powders were used occasional shaking was necessary to keep the powder in suspension. (Sera were more likely to be opalescent when absorbed with powder than when absorbed with extracts.) Although the time of incubation at 40° and amount of powder used varied with the history of the serum and particular organism used, approximately 30 mg. of powder per cc. of serum (or 2 volumes of extract to 1 volume of serum), incubated 4–8 hours, then refrigerated, fell within the range of partial or complete absorption. With this trial as a working basis, variations could be made to obtain desired results. Absorption schedules using refrigeration before and after incubation at 40° showed no consistent differences in results when compared with those of tests run as described.

For control tests, the serum of the normal animal was tested with the same antigens (lower dilutions) against which the immune serum would be run, and against the saline used in preparing these antigens. At least two animals were immunized per organism, and in preliminary experiments each serum was run in duplicate as an additional control. The latter was found unnecessary as different readings of the same serum varied scarcely at all.

## IV. Results

Data here recorded were obtained by the standard procedure outlined in the preceding section. No attempt will be made to present the full protocols of all experiments. All but one of the animals immunized (*F. sporotrichioides*) produced antisera with sufficiently high titers for tests after two series of immunizations. Normal sera of a few animals (13) gave feeble reactions with one or two antigens (notably *F. oxysporum*, *F. dimerum*, and *Gibb. saubinetii*) in the lower dilutions (up to 1:200); normal serum of only one animal reacted with many antigens at 1:200 or above, and consequently this serum was not used. The protocols of tests with normal sera are not given since all that were later used as antisera gave negative reac-

tions in the normal tests. Full reciprocal tests were not made between all organisms because all antigens and antisera were not available simultaneously.

The data of tables II, III, IV, and VI are selected and presented on the following bases:

1. Reactions of $I_2$ sera (produced by *two* standard series of injections).

2. Antiserum with highest titer.

3. Second hour readings (highest titer in duplicate series of tests chosen, when run).

4. The heading Titer represents the highest dilution of the given test antigen with which the given antiserum reacted, including ± reactions.

5. The heading Sum represents the total of reactions of a serum in all dilutions from 1:100 on, ±, 1, 2, 3, and 4 being arbitrarily assigned the numerical values 5, 10, 20, 30, and 40 respectively. Intermediate reactions were assigned intermediate values; for example, 3− = 25.

6. The Percentage heading represents the relative percentage value of the sums of the reactions of a given serum with each test antigen, the homologous reaction being taken at 100%.

7. Homologous values for each serum and antigen are printed in heavy type.

Table II gives the protocols for three pairs of animals immunized with *F. argillaceum*, *F. fructigenum*, and *F. ossicolum* respectively. It shows: (1) typical protocols of tests with antisera (first hour readings are omitted); (2) differences in sera of animals immunized concurrently; (3) the derivation of data of tables III, IV, and VI from full protocols (Titer, Sum, and % appear in their respective columns to the right of each serum).

Tables III and IV record such data synoptically for all organisms investigated, protocols being selected by the criteria previously given. Additional columns at the end of these tables record: (1) the number of antisera per organism from which the cited antiserum was selected; (2) the number of stock test antigen lots prepared per organism; (3) the pH range of these antigen lots (1:100 dilution tested); (4) the most frequent pH of the antigen lots.

## TABLE II

| Organism | F. argillaceum antisera ($I_2$) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum #86* | | | | | | | | Antiserum #87* | | | | | | | | Titer | Sum | % |
| | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | | | |
| F. anthophilum | ± | – | – | – | – | – | – | – | ± | – | – | – | – | – | – | – | 50 | ..... | ..... |
| F. argillaceum | 3 | 3 | 3 | 2 | 1 | ± | ± | – | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ± | **6400** | **185** | **100** |
| F. cepae | ± | – | – | – | – | – | – | – | 1 | 1 | ± | ± | – | – | – | – | 400 | 15 | 8 |
| F. conglutinans | – | – | – | – | – | – | – | – | ± | ± | ± | – | – | – | – | – | 200 | 10 | 5 |
| F. conglutinans var. callestephi | ± | ± | – | – | – | – | – | – | ± | – | – | – | – | – | – | – | 50 | ..... | ..... |
| F. cubense #8 | – | – | – | – | – | – | – | – | ± | ± | ± | – | – | – | – | – | 200 | 10 | 5 |
| F. decemcellulare | ± | ± | – | – | – | – | – | – | ± | ± | – | – | – | – | – | – | 100 | 5 | 2 |
| F. dimerum | – | – | – | – | – | – | – | – | 1 | ± | – | – | – | – | – | – | 100 | 5 | 2 |
| F. fructigenum | ± | ± | – | – | – | – | – | – | ± | 1 | – | – | – | – | – | – | 100 | 5 | 2 |
| F. javanicum | ± | – | – | – | – | – | – | – | 1 | ± | ± | – | – | – | – | – | 200 | 10 | 5 |
| F. lycopersici | – | – | – | – | – | – | – | – | ± | ± | ± | ± | – | – | – | – | 200 | 10 | 5 |
| F. moniliforme | – | – | – | – | – | – | – | – | ± | ± | ± | ± | ± | – | – | – | 400 | 15 | 8 |
| F. nivale | – | – | – | – | – | – | – | – | 1 | ± | ± | ± | ± | – | – | – | 800 | 20 | 10 |
| F. ossicolum | – | – | – | – | – | – | – | – | ± | ± | ± | – | – | – | – | – | 800 | 20 | 10 |
| F. semitectum | – | – | – | – | – | – | – | – | 1 | ± | ± | – | – | – | – | – | 200 | 10 | 3 |
| F. sporotrichioides | – | – | – | – | – | – | – | – | ± | ± | ± | ± | ± | – | – | – | 400 | 20 | 31 |
| F. trichothecioides | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | 0 | 0 | 0 |
| Gibberella saubinetii #259* | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| F. acuminatum | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |

* Saline controls with all sera negative.

TABLE II—*Continued*

| Organism | F. fructigenum antisera ($I_2$) | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum #80* | | | | | | | | | | Antiserum #81* | | | | | | | | | | Titer | Sum | % |
| | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | | | | |
| F. anthophilum | 2 | 2 | 2 | 1 | – | – | – | – | – | | 3 | 3 | 2 | 1 | ± | – | – | – | – | | 800 | 65 | 28 |
| F. argillaceum | – | – | – | – | – | – | – | – | – | | ± | – | – | – | – | – | – | – | – | | 50 | | 13 |
| F. cepae | 2 | 2 | 1 | 1 | – | – | – | – | – | | 3 | 2 | 1 | – | – | – | – | – | – | | 200 | 30 | |
| F. conglutinans | 2 | 2 | 2 | 1 | – | – | – | – | – | | 3 | 3 | 2 | 1 | – | – | – | – | – | | 400 | 60 | 26 |
| F. conglutinans var. callestephi | 2 | 2 | 2 | 1 | ± | – | – | – | – | | 3 | 3 | 2 | 1 | 1 | ± | – | – | – | | 1600 | 75 | 33 |
| F. cubense #8 | 2 | 2 | 2 | 1 | – | – | – | – | – | | 2 | 2 | 2 | 1 | ± | – | – | – | – | | 800 | 55 | 24 |
| F. decemcellulare | ± | ± | – | – | – | – | – | – | – | | 1 | ± | – | – | – | – | – | – | – | | 100 | 5 | 2 |
| F. dimerum | 2 | 2 | 1 | 1 | ± | – | – | – | – | | 4– | 3– | 2 | 1 | ± | – | – | – | – | | 800 | 60 | 26 |
| F. fructigenum | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ± | – | | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ± | | **12,800** | **225** | **100** |
| F. javanicum | 2 | 2 | 2 | 2 | 1 | ± | – | – | – | | 3 | 3 | 3 | 3 | 2 | 2– | 1 | ± | – | | 6400 | 140 | 61 |
| F. lycopersici | 4 | 4 | 3 | 2 | 1 | ± | ± | – | – | | 3 | 3 | 3 | 2 | 1 | 1 | – | – | – | | 1600 | 100 | 44 |
| F. moniliforme | 2 | 2 | 1 | – | – | – | – | – | – | | 3– | 2 | 1 | ± | – | – | – | – | – | | 400 | 35 | 15 |
| F. nivale | – | – | – | – | – | – | – | – | – | | 2 | 2 | 1 | ± | – | – | – | – | – | | 400 | 35 | 15 |
| F. ossicolum | ± | ± | ± | – | – | – | – | – | – | | 1 | 1 | 1 | 1 | ± | – | – | – | – | | 800 | 35 | 15 |
| F. semitectum | 1 | ± | – | – | – | – | – | – | – | | 1 | 1 | 1 | 1 | – | – | – | – | – | | 400 | 30 | 13 |
| F. sporotrichioides | 1 | 1 | ± | – | – | – | – | – | – | | 2 | 2 | 2 | 1 | ± | – | – | – | – | | 800 | 55 | 24 |
| F. trichothecioides | – | – | – | – | – | – | – | – | – | | 2 | 2 | 1 | 1 | 1 | ± | – | – | – | | 1600 | 55 | 24 |
| Gibberella saubinetii #259* | 1 | ± | – | – | – | – | – | – | – | | 3 | 2 | 1 | ± | – | – | – | – | – | | 400 | 35 | 15 |
| F. acuminatum | | | | | | | | | | | | | | | | | | | | | | | |

TABLE II—*Continued*

| Organism | F. ossicolum antisera ($I_3$) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Antiserum #82* | | | | | | | | Antiserum #83* | | | | | | | | | titer | sum | % |
| | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:6400 | 1:12800 | | | |
| F. anthophilum | 3 | 3 | 3 | 3 | 1 | ± | — | — | 4 | 4 | 4 | 4 | 2 | 1 | ± | — | — | 3200 | 155 | 164 |
| F. argillaceum | 2— | ± | — | — | — | — | — | — | ± | — | — | — | — | — | — | — | — | 50 | ..... | ..... |
| F. cepae | 3 | 2 | 1 | ± | — | — | — | — | 3 | 2 | 2— | ± | ± | — | — | — | ..... | 800 | 45 | 47 |
| F. conglutinans | 4 | 3 | 2 | 1 | — | — | — | — | 4 | 3 | 2 | 1 | ± | — | — | — | ..... | 800 | 65 | 68 |
| F. conglutinans var. callestephi | 2 | 3 | 2 | 1 | ± | — | — | — | 3 | 4 | 3 | 2 | 1 | ± | — | — | ..... | 1600 | 105 | 11 |
| F. cubense #8 | 3 | 3 | 2 | 1 | ± | — | — | — | 3 | 3 | 3 | 2 | 1— | — | — | — | ..... | 800 | 85 | 90 |
| F. decemcellulare | 3 | 2 | 1 | ± | ± | — | — | — | 2 | 2 | ± | — | — | — | — | — | ..... | 200 | 25 | 26 |
| F. dimerum | 2 | 2 | 2 | 1 | ± | — | — | — | 2 | 2 | 2 | 1 | ± | — | — | — | ..... | 800 | 55 | 58 |
| F. fructigenum | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ± | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | ± | 12,800 | 225 | 238 |
| F. javanicum | 4 | 4 | 4 | 4 | 3 | 2— | ± | — | 4 | 4 | 4 | 4 | 3 | 1 | ± | — | ..... | 3200 | 165 | 174 |
| F. lycopersici | 4 | 4 | 4 | 2 | 1 | 1— | — | — | 4 | 4 | 4 | 3— | 2 | 1 | ± | — | ..... | 3200 | 140 | 148 |
| F. moniliforme | 3 | 2 | 1 | ± | — | — | — | — | 3— | 2— | 2— | 1 | ± | — | — | — | ..... | 800 | 45 | 47 |
| F. nivale | 2 | 2— | 1 | — | — | — | — | — | 3 | 2 | 1 | ± | — | — | — | — | ..... | 400 | 35 | 37 |
| F. ossicolum | 4 | 4 | 3 | 2 | 1 | ± | — | — | 4 | 4 | 4 | 1 | ± | — | — | — | ..... | **800** | **95** | **100** |
| F. semitectum | 2 | 2 | 1 | 1 | — | — | — | — | 4— | 3 | 3— | 2 | 1— | — | — | — | ..... | 800 | 80 | 84 |
| F. sporotrichioides | 3 | 3 | 2 | 2 | 1 | 1 | — | — | 3 | 3 | 3 | 2 | 1 | ± | — | — | ..... | 1600 | 95 | 100 |
| F. trichothecioides | 4 | 4 | 3 | 1 | ± | — | — | — | 4 | 4 | 3 | 2 | 1 | ± | — | — | ..... | 1600 | 105 | 111 |
| Gibberella saubinetii #259* | 3 | 2 | 1 | ± | — | — | — | — | 3 | 3— | 3— | 1 | — | — | — | — | ..... | 400 | 60 | 63 |
| F. acuminatum | 4 | 4 | 3 | 2 | ± | — | — | — | 4 | 4 | 3 | 1 | ± | — | — | — | ..... | ..... | ..... | ..... |

## TABLE III

### IMMUNE SERA

| ORGANISM | 1 | | | 2 | | | 3 | | | 4* | | | 5 | | | 6 | | | 7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % |
| 1 F. anthophilum | **3200** | **145** | **100** | 50 | | | 400 | 30 | 30 | 1600 | 60 | 51 | 400 | 20 | 10 | 1600 | 130 | 72 | 200 | 20 | 50 |
| 2 F. argillaceum | 200 | 20 | 13 | **6400** | **185** | **100** | 200 | 10 | 10 | 800 | 25 | 21 | 0 | 0 | 0 | 200 | 20 | 10 | 0 | 0 | 0 |
| 3 F. cepae | 400 | 25 | 17 | 400 | 15 | 8 | **1600** | **100** | **100** | 1600 | 45 | 79 | 400 | 50 | 27 | 1600 | 105 | 56 | 0 | 0 | 0 |
| 4 F. conglutinans | 400 | 40 | 27 | 200 | 10 | 5 | 800 | 55 | 55 | **6400** | **115** | **100** | 3200 | 100 | 55 | 3200 | 155 | 83 | 1600 | 75 | 187 |
| 5 F. conglutinans var. callistephi | 1600 | 70 | 48 | 50 | | | 800 | 100 | 100 | 6400 | 115 | 100 | **12,800** | **180** | **100** | 6400 | 205 | 110 | 1600 | 75 | 187 |
| 6 F. cubense #8 | 800 | 55 | 37 | 200 | 10 | 5 | 800 | 45 | 45 | 1600 | 65 | 55 | 3200 | 105 | 56 | **3200** | **180** | **100** | 1600 | 75 | 187 |
| 7 F. decemcellulare | 100 | 5 | 3 | 100 | 5 | 2 | 100 | 5 | 5 | 800 | 40 | 34 | 50 | | | 200 | 20 | 10 | **800** | **40** | **100** |
| 8 F. dimerum | 400 | 30 | 20 | 100 | 5 | 2 | 400 | 25 | 25 | 3200 | 55 | 47 | 100 | 10 | 5 | 800 | 80 | 43 | 400 | 40 | 100 |
| 9 F. fructigenum | 3200 | 110 | 75 | 100 | 5 | 2 | 800 | 50 | 50 | 3200 | 100 | 86 | 6400 | 130 | 60 | 19,200 | 175 | 94 | 3200 | 105 | 262 |
| 10 F. javanicum | 1600 | 60 | 41 | 200 | 10 | 5 | 800 | 35 | 35 | 6400 | 110 | 95 | 100 | 5 | 2 | 12,800 | 205 | 110 | 3200 | 100 | 250 |
| 11 F. lycopersici | 3200 | 90 | 62 | 200 | 10 | 5 | 800 | 80 | 80 | 3200 | 70 | 60 | 3200 | 155 | 85 | 3200 | 180 | 100 | 1600 | 85 | 212 |
| 12 F. moniliforme | 400 | 35 | 24 | 200 | 15 | 8 | 800 | 45 | 45 | 1600 | 55 | 47 | 800 | 65 | 35 | 1600 | 110 | 59 | 400 | 25 | 62 |
| 13 F. nivale | 50 | | | 800 | 20 | 10 | 200 | 15 | 15 | 800 | 35 | 29 | 50 | | | 400 | 50 | 27 | 100 | 5 | 12 |
| 14 F. ossicolum | 200 | 10 | 6 | 800 | 20 | 10 | 800 | 25 | 25 | 3200 | 65 | 55 | 50 | | | 1600 | 105 | 56 | 100 | 5 | 12 |
| 15 F. oxysporum | | | | | | | | | | | | | | | | | | | | | |
| 16 F. semitectum | 100 | 5 | 3 | 200 | 10 | 5 | 200 | 15 | 15 | 800 | 40 | 34 | 50 | | | 400 | 35 | 18 | 50 | | |
| 17 F. sporotrichioides | 800 | 45 | 31 | 400 | 20 | 10 | 800 | 35 | 35 | 1600 | 55 | 47 | 200 | 10 | 5 | 1600 | 120 | 64 | 400 | 30 | 75 |
| 18 F. theobromae | | | | | | | | | | | | | | | | | | | | | |
| 19 F. trichothecioides | 200 | 15 | 10 | 0 | 0 | 0 | 800 | 30 | 30 | 1600 | 80 | 68 | 50 | | | 3200 | 105 | 56 | 200 | 20 | 50 |
| 20 Gibberella saubinetii #259 | 200 | 15 | 10 | 100 | 5 | 2 | 800 | 20 | 20 | 1600 | 35 | 29 | 200 | 20 | 10 | 6400 | 95 | 51 | 400 | 45 | 112 |
| 21 Hypomyces ipomoeae | | | | | | | | | | | | | | | | | | | | | |
| 22 Cylindrocarpon album | | | | | | | | | | | | | | | | | | | | | |
| 23 Ramularia sp. | | | | | | | | | | | | | | | | | | | | | |
| 24 Neurospora tetrasperma A+B | | | | | | | | | | | | | | | | | | | | | |
| 25 Sclerotinia sclerotiorum | | | | | | | | | | | | | | | | | | | | | |
| 26 F. acuminatum | | | | | | | | | | | | | | | | | | | | | |
| 27 F. sambucinum | | | | | | | 800 | 50 | 50 | | | | | | | | | | | | |
| 28 Gibberella saubinetii (English strain) | | | | | | | | | | | | | | | | | | | | | |

* Sum represents total of reactions in all dilutions from 1:200 on.

TABLE III—*Continued*

| Organism | 8 | | | 9 | | | 10 | | | 11 | | | 12 | | | 13 | | | 14 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % |
| 1 F. anthophilum | 100 | 15 | 14 | 800 | 65 | 28 | 800 | 55 | 31 | 1600 | 55 | 39 | 400 | 25 | 33 | 800 | 65 | 61 | 3200 | 155 | 164 |
| 2 F. argillaceum | 0 | 0 | .... | 0 | 0 | 0 | 400 | 35 | 20 | 100 | 5 | 3 | 200 | 15 | 19 | 100 | 10 | 9 | 50 | .... | .... |
| 3 F. cepae | 200 | 20 | 19 | 200 | 30 | 13 | 200 | 20 | 11 | 1600 | 105 | 75 | 400 | 35 | 46 | 400 | 25 | 23 | 800 | 45 | 47 |
| 4 F. conglutinans | 1600 | 55 | 52 | 400 | 60 | 26 | 200 | 10 | 5 | 800 | 65 | 46 | 3200 | 100 | 132 | 50 | .... | .... | 800 | 65 | 68 |
| 5 F. conglutinans var. callistephi | 1600 | 75 | 72 | 1600 | 75 | 33 | 400 | 40 | 23 | 3200 | 180 | 129 | 3200 | 110 | 145 | 800 | 30 | 28 | 1600 | 105 | 111 |
| 6 F. cubense #8 | 800 | 70 | 67 | 800 | 55 | 24 | 400 | 30 | 17 | 3200 | 150 | 108 | 3200 | 85 | 112 | 50 | .... | .... | 800 | 85 | 90 |
| 7 F. decemcellulare | 100 | 5 | 4 | 100 | 5 | 2 | 200 | 15 | 8 | 100 | 5 | 3 | 0 | 0 | 0 | 200 | 15 | 14 | 200 | 25 | 26 |
| 8 F. dimerum | **1600** | **105** | **100** | 800 | 60 | 26 | 400 | 20 | 11 | 200 | 20 | 14 | 800 | 25 | 33 | 200 | 30 | 28 | 800 | 55 | 58 |
| 9 F. fructigenum | 3200 | 115 | 110 | **12,800** | **225** | **100** | 6400 | 155 | 89 | 6400 | 145 | 104 | 6400 | 120 | 158 | 800 | 65 | 61 | 12,800 | 225 | 238 |
| 10 F. javanicum | 1600 | 75 | 72 | 6400 | 140 | 61 | **3200** | **170** | **100** | 3200 | 85 | 61 | 1600 | 75 | 100 | 800 | 40 | 38 | 3200 | 165 | 174 |
| 11 F. lycopersici | 1600 | 105 | 100 | 1600 | 100 | 44 | 3200 | 115 | 66 | **3200** | **140** | **100** | 3200 | 115 | 151 | 400 | 25 | 23 | 3200 | 140 | 148 |
| 12 F. moniliforme | 400 | 40 | 38 | 400 | 35 | 15 | 200 | 15 | 8 | 800 | 65 | 46 | **1600** | **75** | **100** | 400 | 35 | 33 | 800 | 45 | 47 |
| 13 F. nivale | 200 | 20 | 19 | 400 | 35 | 15 | 200 | 10 | 5 | 50 | .... | .... | 200 | 20 | 26 | **1600** | **105** | **100** | 400 | 35 | 37 |
| 14 F. ossicolum | 400 | 25 | 24 | 800 | 35 | 15 | 200 | 20 | 11 | 100 | 20 | 14 | 400 | 20 | 26 | 400 | 35 | 33 | **800** | **95** | **100** |
| 15 F. oxysporum | .... | .... | .... | .... | .... | .... | 200 | 15 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 16 F. semitectum | 200 | 15 | 14 | 400 | 30 | 13 | 200 | 25 | 14 | 50 | .... | .... | 200 | 10 | 13 | 800 | 80 | 75 | 800 | 80 | 84 |
| 17 F. sporotrichioides | 400 | 65 | 62 | 800 | 55 | 24 | 400 | 20 | 11 | 800 | 45 | 32 | 400 | 30 | 39 | 400 | 15 | 14 | 1600 | 95 | 100 |
| 18 F. theobromae | .... | .... | .... | .... | .... | .... | 1600 | 65 | 37 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 19 F. trichothecioides | 400 | 30 | 28 | 1600 | 55 | 24 | 400 | 25 | 14 | 400 | 30 | 21 | 200 | 10 | 13 | 200 | 15 | 14 | 1600 | 105 | 111 |
| 20 Gibberella saubinetii #259 | 800 | 45 | 43 | 400 | 35 | 15 | 800 | 20 | 11 | 400 | 35 | 25 | 1600 | 80 | 105 | 400 | 35 | 33 | 400 | 60 | 63 |
| 21 Hypomyces ipomoeae | .... | .... | .... | .... | .... | .... | 3200 | 155 | 89 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 22 Cylindrocarpon album | .... | .... | .... | .... | .... | .... | 400 | 30 | 17 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 23 Ramularia sp. | .... | .... | .... | .... | .... | .... | 50 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 24 Neurospora tetrasperma A+B | .... | .... | .... | .... | .... | .... | 0 | 0 | 0 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 25 Sclerotinia sclerotiorum | .... | .... | .... | .... | .... | .... | 0 | 0 | 0 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 26 F. acuminatum | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | 800 | 85 | 90 |
| 27 F. sambucinum | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 28 Gibberella saubinetii (English strain) | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |

TABLE III—*Continued*

| Organism | 15 | | | 16 | | | 17 | | | 18 | | | 19 | | | 20 | | | 21 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % |
| 1 F. anthophilum | 100 | 5 | 7 | 200 | 15 | 23 | 1600 | 130 | 78 | 1600 | 100 | 74 | 400 | 50 | 50 | 200 | 15 | 14 | 1600 | 40 | 26 |
| 2 F. argillaceum | 0 | 0 | 0 | 50 | .... | .... | 800 | 55 | 33 | 200 | 15 | 11 | 0 | 0 | 0 | 100 | 5 | 4 | 100 | 5 | 3 |
| 3 F. cepae | 200 | 35 | 49 | 200 | 25 | 38 | 1600 | 140 | 84 | 400 | 25 | 18 | 200 | 15 | 15 | 200 | 15 | 14 | 1600 | 35 | 23 |
| 4 F. conglutinans | 50 | .... | .... | 200 | 15 | 23 | 3200 | 175 | 106 | 200 | 20 | 14 | 800 | 65 | 65 | 400 | 35 | 32 | 0 | 0 | 0 |
| 5 F. conglutinans var. callistephi | 400 | 15 | 21 | 800 | 70 | 107 | 6400 | 205 | 124 | 400 | 50 | 37 | 3200 | 125 | 125 | 400 | 35 | 32 | 1600 | 35 | 23 |
| 6 F. cubense #8 | 0 | 0 | 0 | 200 | 35 | 53 | 6400 | 185 | 109 | 800 | 55 | 40 | 1600 | 95 | 95 | 400 | 35 | 32 | 1600 | 50 | 33 |
| 7 F. decemcellulare | 50 | .... | .... | 50 | .... | .... | 800 | 100 | 60 | 400 | 30 | 22 | 200 | 15 | 15 | 100 | 10 | 9 | 200 | 20 | 13 |
| 8 F. dimerum | 200 | 15 | 21 | 800 | 100 | 154 | 3200 | 145 | 87 | 800 | 45 | 33 | 1600 | 105 | 105 | 400 | 35 | 32 | 400 | 25 | 16 |
| 9 F. fructigenum | 200 | 15 | 21 | 6400 | 185 | 284 | 12,800 | 215 | 130 | 6400 | 175 | 129 | 3200 | 130 | 130 | 800 | 60 | 56 | 1600 | 105 | 69 |
| 10 F. javanicum | 200 | 10 | 14 | 3200 | 165 | 254 | 6400 | 145 | 87 | 3200 | 175 | 129 | 1600 | 110 | 110 | 800 | 45 | 42 | 1600 | 140 | 92 |
| 11 F. lycopersici | 1600 | 40 | 56 | 3200 | 145 | 223 | 3200 | 190 | 115 | 1600 | 130 | 96 | 3200 | 145 | 145 | 1600 | 105 | 100 | 3200 | 95 | 62 |
| 12 F. moniliforme | 400 | 35 | 49 | 400 | 45 | 69 | 3200 | 150 | 90 | 400 | 35 | 25 | 800 | 90 | 90 | 400 | 35 | 32 | 400 | 20 | 13 |
| 13 F. nivale | 400 | 25 | 35 | 400 | 55 | 84 | 1600 | 120 | 72 | 200 | 15 | 11 | 400 | 60 | 60 | 800 | 45 | 42 | 800 | 30 | 19 |
| 14 F. ossicolum | 400 | 10 | 14 | 800 | 80 | 123 | 6400 | 170 | 103 | 800 | 40 | 29 | 800 | 100 | 100 | 400 | 25 | 23 | 1600 | 45 | 29 |
| 15 F. oxysporum | **800** | **70** | **100** | .... | .... | .... | .... | .... | .... | 800 | 30 | 22 | .... | .... | .... | .... | .... | .... | 6400 | 60 | 39 |
| 16 F. semitectum | 200 | 15 | 21 | **800** | **65** | **100** | 3200 | 15 | 90 | 1600 | 55 | 40 | 800 | 45 | 45 | 200 | 20 | 18 | 3200 | 60 | 39 |
| 17 F. sporotrichioides | 400 | 30 | 42 | 800 | 65 | 100 | **3200** | **165** | **100** | 400 | 40 | 29 | 400 | 60 | 60 | 400 | 45 | 42 | 3200 | 70 | 46 |
| 18 F. theobromae | .... | .... | .... | .... | .... | .... | .... | .... | .... | **1600** | **135** | **100** | .... | .... | .... | .... | .... | .... | 400 | 45 | .... |
| 19 F. trichothecioides | 100 | 5 | 7 | 400 | 45 | 69 | 3200 | 135 | 87 | 400 | 60 | 44 | **800** | **100** | **100** | 800 | 45 | 42 | 1600 | 55 | 36 |
| 20 Gibberella saubinetii #259 | 0 | 0 | 0 | 800 | 70 | 107 | 1600 | 80 | 48 | 800 | 40 | 29 | 800 | 80 | 80 | **1600** | **105** | **100** | 800 | 25 | 16 |
| 21 Hypomyces ipomoeae | 0 | 0 | 0 | .... | .... | .... | .... | .... | .... | 3200 | 180 | 133 | .... | .... | .... | .... | .... | .... | **3200** | **150** | **130** |
| 22 Cylindrocarpon album | 0 | 0 | 0 | .... | .... | .... | .... | .... | .... | 1600 | 55 | 40 | .... | .... | .... | .... | .... | .... | 6400 | 55 | 36 |
| 23 Ramularia sp. | 50 | .... | .... | .... | .... | .... | .... | .... | .... | 800 | 55 | 40 | .... | .... | .... | .... | .... | .... | 200 | 10 | 6 |
| 24 Neurospora tetrasperma A+B | 0 | 0 | 0 | .... | .... | .... | .... | .... | .... | 50 | .... | .... | .... | .... | .... | .... | .... | .... | 0 | 0 | 0 |
| 25 Sclerotinia sclerotiorum | 100 | 10 | 14 | .... | .... | .... | .... | .... | .... | 50 | .... | .... | .... | .... | .... | .... | .... | .... | 400 | 15 | 10 |
| 26 F. acuminatum | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| 27 F. sambucinum | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | 800 | 45 | 45 | .... | .... | .... | .... | .... | .... |
| 28 Gibberella saubinetii (English strain) | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | 800 | 65 | 61 | .... | .... | .... |

TABLE III—*Continued*

| Organism | 22 | | | 23 | | | 24 | | | 25 | | | No. immune sera per organism | No. stock antigen lots prepared | pH range of stock antigen lots | Most frequent pH of stock antigen lots |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | titer | sum | % | titer | sum | % | titer | sum | % | titer | sum | % | | | | |
| 1 F. anthophilum | 400 | 15 | 33 | 0 | 0 | 0 | 400 | 20 | 23 | 0 | 0 | 0 | 2 | 16 | 4.9-6.0 | 5.4 |
| 2 F. argillaceum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 25 | 5.4-6.4 | 6.1 |
| 3 F. cepae | 0 | 0 | 0 | 100 | 5 | 16 | 800 | 50 | 59 | 0 | 0 | 0 | 3 | 18 | 5.1-6.1 | 5.9 |
| 4 F. conglutinans | 0 | 0 | 0 | 0 | 0 | 0 | 1600 | 35 | 41 | 0 | 0 | 0 | 8 | 21 | 6.0-6.6 | 6.2 |
| 5 F. conglutinans var. callistephi | 0 | 0 | 0 | 0 | 0 | 0 | 3200 | 75 | 88 | 0 | 0 | 0 | 8 | 27 | 5.7-6.4 | 5.9 |
| 6 F. cubense #8 | 50 | ..... | ..... | 0 | 0 | 0 | 3200 | 60 | 70 | 0 | 0 | 0 | 5 | 27 | 6.6-7.1 | 6.8 |
| 7 F. decemcellulare | 0 | 0 | 0 | 0 | 0 | 0 | 400 | 20 | 23 | 0 | 0 | 0 | 2 | 17 | 5.7-6.1 | 5.9 |
| 8 F. dimerum | 100 | 5 | 11 | 100 | 10 | 33 | 3200 | 45 | 53 | 100 | 5 | 2 | 2 | 17 | 5.3-6.1 | 5.8 |
| 9 F. fructigenum | 50 | ..... | ..... | 50 | ..... | ..... | 400 | 20 | 23 | 0 | 0 | 0 | 2 | 29 | 6.5-7.0 | 6.7 |
| 10 F. javanicum | 100 | 5 | 11 | 200 | 10 | 33 | 1600 | 55 | 64 | 50 | ..... | ..... | 9 | 33 | 5.6-6.5 | 6.1 |
| 11 F. lycopersici | 200 | 20 | 44 | 0 | 0 | 0 | 800 | 60 | 70 | 0 | 0 | 0 | 6 | 28 | 5.5-6.0 | 5.7 |
| 12 F. moniliforme | 0 | 0 | 0 | 200 | 10 | 33 | 1600 | 45 | 53 | 0 | 0 | 0 | 2 | 26 | 5.1-5.6 | 5.3 |
| 13 F. nivale | 0 | 0 | 0 | 0 | 0 | 0 | 800 | 30 | 35 | 0 | 0 | 0 | 3 | 18 | 5.1-5.7 | 5.3 |
| 14 F. ossicolum | 0 | 0 | 0 | 0 | 0 | 0 | 1600 | 50 | 59 | 0 | 0 | 0 | 2 | 17 | 5.1-5.6 | 5.3 |
| 15 F. oxysporum | 400 | 25 | 55 | 200 | 15 | 49 | 400 | 30 | 35 | 400 | 20 | 14 | 2 | 12 | 5.6-5.7 | 5.6 |
| 16 F. semitectum | 0 | 0 | 0 | 0 | 0 | 0 | 200 | 10 | 11 | 0 | 0 | 0 | 2 | 18 | 5.3-5.9 | 5.6 |
| 17 F. sporotrichioides | 0 | 0 | 0 | 0 | 0 | 0 | 800 | 40 | 47 | 0 | 0 | 0 | 5 | 17 | 4.9-5.6 | 5.1 |
| 18 F. theobromae | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 4 | 7 | 5.5-6.0 | 5.9 |
| 19 F. trichothecioides | 0 | 0 | 0 | 0 | 0 | 0 | 800 | 40 | 47 | 0 | 0 | 0 | 5 | 20 | 5.6-6.7 | 5.8 |
| 20 Gibberella saubinetii #259 | 0 | 0 | 0 | 0 | 0 | 0 | 800 | 35 | 41 | 0 | 0 | 0 | 4 | 18 | 5.1-5.8 | 5.4 |
| 21 Hypomyces ipomoeae | 0 | 0 | 0 | 0 | 0 | 0 | 3200 | 55 | 64 | 0 | 0 | 0 | 8 | 18 | 5.6-6.1 | 5.9 |
| 22 Cylindrocarpon album | **800** | **45** | **100** | 200 | 15 | 49 | 1600 | 45 | 53 | 800 | 30 | 22 | 2 | 2 | 5.5-6.6 | ..... |
| 23 Ramularia sp. | 50 | ..... | ..... | **200** | **30** | **100** | 400 | 25 | 29 | 50 | ..... | ..... | 2 | 2 | 5.7-5.8 | ..... |
| 24 Neurospora tetrasperma A+B | 0 | 0 | 0 | 0 | 0 | 0 | **1600** | **85** | **100** | 100 | 5 | 3 | 2 | 5 | 5.6-6.2 | 6.1 |
| 25 Sclerotinia sclerotiorum | 100 | 10 | 22 | 100 | 5 | 16 | 100 | 15 | 18 | **6400** | **135** | **100** | 6 | 21 | 5.0-6.2 | ..... |
| 26 F. acuminatum | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 2 | 5.7-6.0 | ..... |
| 27 F. sambucinum | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 2 | 5.7-6.0 | ..... |
| 28 Gibberella saubinetii (English strain) | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | 3 | 5.7-5.8 | ..... |

## TABLE IV

### IMMUNE SERUM

| ORGANISM | 6A | | | 31 | | | 32 | | | 24 | | | 33 | | | 34 | | | 25A | | | No. IMMUNE SERA PER ORGANISM | No. STOCK ANTIGEN LOTS PREPARED | pH RANGE OF STOCK ANTIGEN LOTS | MOST FREQUENT pH OF STOCK ANTIGEN LOTS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | TITER | SUM | % | | | | |
| 29 F. cubense #4 | 12,800 | 240 | 111 | | | | | | | | | | | | | | | | | | | | 2 | | |
| 30 F. cubense #5 | 6,400 | 160 | 74 | | | | | | | | | | | | | | | | | | | | 2 | | |
| 6a F. cubense #8 | **12,800** | **215** | **100** | | | | | | | | | | | | | | | | | | | 5 | 27 | 6.6-7.1 | 6.8 |
| 31 Neurospora tetrasperma A | | | | **1600** | **75** | **100** | 100 | 5 | 16 | 1600 | 90 | 105 | | | | | | | | | | 5 | 10 | 5.5-6.0 | 6.0 |
| 32 N. tetrasperma B | | | | 800 | 55 | 72 | **200** | **30** | **100** | 1600 | 105 | 123 | | | | | | | | | | 5 | 10 | 5.6-6.9 | 5.7 |
| 24 N. tetrasperma A+B | | | | 1600 | 85 | 112 | 200 | 10 | 33 | **1600** | **85** | **100** | | | | | | | | | | 2 | 5 | 5.6-6.2 | 6.1 |
| 33 S. fructicola #1 | | | | | | | | | | | | | **800** | **40** | **100** | 100 | 10 | 22 | 1600 | 50 | 35 | 2 | 6 | 5.6-5.9 | 5.7 |
| 34 S. fructicola #2 | | | | | | | | | | | | | 800 | 35 | 87 | **1600** | **45** | **100** | 400 | 25 | 17 | 3 | 6 | 5.9-6.2 | 6.0 |
| 25a S. sclerotiorum | | | | | | | | | | | | | 3200 | 65 | 162 | 1600 | 60 | 133 | **3200** | **140** | **100** | 6 | 21 | 5.0-6.2 | |

Protocols of table II represent three of the types of reaction found upon analysis of the serological data. Tables III and IV show additional instances, including limited tests, of these and other types, which are summarized as follows:

1. Antigen and serum specific... F. argillaceum, F. oxysporum, S. sclerotiorum
2. Antigen and serum specific except in 2–3 cases..... F. fructigenum, F. anthophilum, F. nivale, F. cepae, F. conglutinans, F. dimerum, F. conglutinans var. callistephi, Gibb. saubinetii no. 259, Hypomyces ipomoeae, Neurospora tetrasperma A+B, N. tetrasperma B, Ramularia sp., S. fructicola no. 1, S. fructicola no .2
3. Antigen specific; serum non-specific..... F. theobromae, F. sporotrichioides
4. Antigen non-specific; serum specific..... F. javanicum, Cylindrocarpon album
5. Antigen and serum non-specific..... F. ossicolum, F. trichothecioides

The remaining unlisted organisms fall into a group of intermediate specificity for both serum and antigen. The most non-specific serum was that of *F. decemcellulare*, which reacted higher with 8 out of 20 heterologous antigens than with its homologous antigen. The most non-specific antigen was that of *F. lycopersici*, which reacted higher with 7 out of 24 heterologous antisera than with its homologous antiserum. The sera against which most test antigens gave non-specific reactions were *F. cubense* no. 8 and *F. sporotrichioides*. The test antigens against which most sera gave non-specific reactions were *F. fructigenum*, *F. lycopersici*, *F. conglutinans* var. *callistephi*, and *F. javanicum*. Several other antigens and sera gave non-specific reactions, but only in one or two cases each.

The specific precipitability of saline extracts of the *Sclerotinia*

species on the one hand, and of the *Fusarium*, *Cylindrocarpon*, *Ramularia*, and *Neurospora* species on the other, was sufficiently marked to permit differentiation of members of the Pezizales from members of the Hypocreales. Among members of the Hypocreales this specificity was sufficient to permit ready differentiation between the groups mentioned. Differentiation between members of the *Fusarium* group and *Neurospora* was less marked than that between the *Fusarium* group, *Cylindrocarpon*, and *Ramularia*.

Although the *Sclerotinia* species gave marked cross reactions, differences exist in the precipitabilities of the extracts of *S. sclerotiorum* and *S. fructicola*. Neither the plus and minus strains of *N. tetrasperma* nor the two strains of *S. fructicola* could be separated. Similarly the extracts of the *Fusarium* species gave such marked cross reactions (excepting *F. argillaceum*) that sharp differentiation between them was not possible.

Hence the test did not readily differentiate all entities which are separable by morphological and physiological criteria such as host and symptom specificity. However, although many of the organisms studied were not separable by the precipitin test, a consideration of all reactions of every organism leads to the conclusion that each organism is a distinct serological entity.

### V. Supplementary tests and results

These experiments, undertaken to investigate failure of the precipitin test to differentiate fungi separable by morphological and physiological criteria, consisted of: (1) Tests with antisera to determine the effect of additional immunizing series after the $I_2$ series; decline of titer *in vivo* and *in vitro;* effect of long rest periods and subsequent reimmunizations. (2) Study of the pH of immune sera and of test antigen lots; extraction and dilution of the latter in buffered saline solutions of different pH values to find the effect of different hydrogen-ion concentrations upon the reaction between a given test antigen and serum. (3) Determination of the total nitrogen content of powders and extracts by micro-Kjeldahl tests (4) and study of the effect of equivalent and different nitrogen contents of inject and test antigens upon the titer of given antisera. (4) Precipitin absorption tests.

1. ANTISERA.—It was found, in general, that if the titers for homologous and heterologous test antigens were high in an antiserum prepared by two series of injections ($I_2$), a third series of injections ($I_3$) would raise both titers slightly. If, however, the homologous reaction was high and the heterologous reaction low, the titer of the latter was raised more, proportionately, than that of the former after the animal had received a third series of injections. Additional series in either type bring about a gradual rise in titer of both homologous and heterologous reactions. This rise is sometimes erratic for a given antigen against different lots of the same serum. Thus an antigen may give a lower reaction against an antiserum tested after five series of injections than it did against the same serum tested after four series of injections.

Incidental observations were made relative to the titer of antisera stored in and out of the animal body. In general, when several weeks had elapsed after the final series of injections, the titer fell more rapidly *in vivo* than *in vitro*. For example, an antiserum of *F. fructigenum* prepared with three series of injections and stored *in vitro* for six weeks gave a higher titer against heterologous antigens than did serum taken from the animal four weeks after immunization. Homologous reactions in the two were the same. In another sample of this serum kept *in vivo* six weeks and then tested, the heterologous reactions were almost gone and the homologous titer was decidedly lower than in the two samples just cited.

The greater the number of immunization series preceding an interval of rest, the less drastic is the drop in titer during that period. Antiserum of an animal immunized with two series of injections may still show approximately half of its titer after 18 weeks of storage *in vitro*. Many sera, however, showed substantial diminution in titer after 4 or 5 weeks of storage. After periods of 10 or 12 weeks tests might reveal the following trends in titer change: (1) nearly all reactions ceasing or reduced to $\pm$ in the dilution 1:200; (2) strong homologous and heterologous reactions disappearing while weak heterologous reactions remained apparently unchanged; (3) a homologous reaction persisting longer than an equally high heterologous reaction.

Record was made also of the effect of additional series of injections

received a long interval after the two initial series. An antiserum prepared by two such series after a 16-week interval showed a strong advance in titer for all antigens, much greater than that exhibited after the two initial series of injections. Injection with a third series produced no great change from that achieved by the two preceding series, heterologous reactions still showing a proportionately higher rise than homologous reactions. After 16 weeks' storage following reimmunization, this serum showed less drop in titer than it had in the same interval following the two initial series of injections.

The conclusion was drawn that the number and intervals of injection series used for standard immunization were not responsible for the non-specific reactions noted.

2. EFFECT OF HYDROGEN-ION CONCENTRATION ON SPECIFICITY OF TESTS.—Experiments were made to determine whether the hydrogen-ion concentration of antisera and test antigens was a factor in the non-specific reactions obtained. All readings were made electrometrically on the 1:100 dilution of test antigens and on undiluted serum. As an additional check some readings were made colorimetrically on the same material.

Since the pH of the antisera tested ranged from 7.3 to 7.8, 7.5–7.6 being the most frequent value, it was concluded that the hydrogen-ion concentration of antisera was not a critical factor. The pH of the stock saline used in preparation of antigens ranged from 5.5 to 6.7, approximately 5.8 being the most frequent value.

Tables III and IV show that considerable variations occurred in the pH of different test antigens, and in the pH of different lots of the same test antigen. They also show that although all such antigens were acid, none was extremely so. The tables indicate that there is no consistent correlation between the potencies of test antigens and their hydrogen-ion concentration. Thus, although the most frequent pH of test antigen lots of *F. conglutinans* var. *callistephi* and *F. decemcellulare* is the same, the former antigen has a much stronger reaction against all sera than has the latter.

To check this preliminary conclusion, detailed studies were made with nine species of *Fusarium* and with *Sclerotinia sclerotiorum*. The control lots were prepared according to the standard procedure, with unbuffered saline of approximately pH 6.0. Comparable lots of test

antigen were prepared using 0.85% NaCl solution buffered to give pH values of 4.4, 5.0, 6.0, 7.0, and 7.6. Modifications of test antigen solutions were of three types: (1) powder extracted with saline buffered to pH 4.4 and dilutions made with 4.4 saline; (2) powder extracted with unbuffered saline (6.0) and dilutions made with 4.4 saline; (3) powder extracted with saline buffered to 4.4 and dilutions made with unbuffered NaCl. This series was repeated using pH values 5.0, 6.0, 7.0, and 7.6.

The first type of extraction and dilution was applied to all nine organisms, the second to many, and the third to only a few. Homologous tests were run with each antigen, and in most cases, tests with several heterologous sera, so that in all, nine test antigens, each with 6 to 16 modifications, were run against several members of a group of 18 antisera.

Titers obtained with buffered preparations of test antigens showed little variation from those obtained with unbuffered preparations of stock antigens (standard procedure). Greatest deviation was noted in tests with antigens buffered to pH 4.4 and 5.0. These titers were usually lower than those of the other buffered lots. Atypical behavior was especially noticeable at pH 4.4, such as: (1) reaction occurring when there was none in the other antigen preparations; (2) very weak reactions extending without gradation to the limit of the dilutions tested; (3) "rings" in the control tube (not with all sera).

Powders extracted and diluted with 4.4 and 5.0 buffer lots were slightly less potent than those extracted with unbuffered saline (pH 6.0) and diluted with saline buffered to pH 4.4 and 5.0.

Since these anomalous reactions occurred in a pH range below that of test antigens prepared by the standard method, it is concluded that the variations in titer and non-specific reactions observed were not materially affected by the pH of the saline used in preparation of the stock antigens.

3. MICRO-KJELDAHL TESTS.—On the assumption that quantitative differences in the nitrogen content of the extracts and powders might be a factor in the non-specific reactions noted, a series of total nitrogen determinations was made using the modified KOCH and MCMEEKIN (4) micro-Kjeldahl test. Several representative fungus

powders were analyzed. Samples of the following were used: saline extracts prepared from powder extracted with petrol ether; saline extracts prepared from untreated powders; powder extracted with petrol ether; untreated powders. The results are given in table V.

TABLE V

| Organism | Milligrams of nitrogen in | | | |
|---|---|---|---|---|
| | 1 cc. 1:50 saline extract of powder | 1 cc. 1:50 saline extract of ligroin-treated powder | 10 mg. of untreated powder | 10 mg. of ligroin-treated powder |
| F. anthophilum | ..... | ..... | 0.35 | ..... |
| F. argillaceum | 0.27 | ..... | ..... | ..... |
| F. cepae | ..... | ..... | 0.48 | ..... |
| F. conglutinans | 0.15 | ..... | 0.45 | ..... |
| F. conglutinans var. callistephi | 0.10–0.20* | ..... | 0.51 | ..... |
| F. cubense #8 | 0.10–0.22 | ..... | 0.45 | ..... |
| F. decemcellulare | 0.15 | ..... | 0.33 | ..... |
| F. dimerum | ..... | ..... | 0.39 | ..... |
| F. fructigenum | 0.15–0.53 | 0.49 | 0.51–0.54 | 0.27 |
| F. javanicum | 0.34 | 0.35 | 0.49 | 0.49 |
| F. lycopersici | 0.20 | ..... | 0.50 | ..... |
| F. oxysporum | 0.59 | ..... | 0.61 | ..... |
| F. theobromae | 0.33 | 0.43 | 0.39 | 0.55 |
| Gibb. saubinetii #259 | 0.14 | ..... | ..... | ..... |
| Gibb. saubinetii (English) | 0.21 | ..... | ..... | ..... |
| Hypomyces ipomoeae | 0.41 | 0.44 | 0.46 | 0.49 |
| Cylindrocarpon album | 0.25 | ..... | 0.40 | ..... |
| Ramularia sp. | 0.22 | ..... | 0.29 | ..... |
| Neurospora tetrasperma A+B | 0.46 | ..... | 0.44 | ..... |
| S. sclerotiorum | 0.10 | 0.08 | 0.15 | 0.11 |

* Two numbers indicate range where difference in tests is great.

The nitrogen content of the powders and saline extracts varied considerably. A comparison of tables III, IV, and V, however, does not show a significant correlation between the potency of a given antigen or antiserum and the nitrogen content of that organism's powder or extract. Thus, test antigens of *F. decemcellulare* which produced relatively low titers with all antisera contained as much nitrogen as those of *F. conglutinans* which produced relatively high titers with these antisera.

To check this observation, experiments were run with test and inject antigens of closely related organisms standardized against an arbitrary unit to contain the same quantity of nitrogen. The cultures selected for the test are designated *Hypomyces ipomoeae* and *F. theobromae*, and supposedly are two cultures of the same taxonomic entity, *F. javanicum* var. *theobromae*, which is supposed to be the conidial stage of *Hypomyces ipomoeae*. The culture designated *F. javanicum* supposedly is the imperfect species of which the culture of *F. theobromae* is a variety.

Antisera were prepared of *Hypomyces ipomoeae*, *F. theobromae*, and *F. javanicum*. Test antigens were prepared for each and for *S. sclerotiorum* and *F. fructigenum*. Control antisera for each organism were produced by two series of injections, each series containing 0.3 gm. of powder (standard immunization). Another set of antisera were prepared per organism with two series of injections, each series containing amounts of powder equivalent in nitrogen to a given unit. Similarly, standardized test antigen lots were prepared. For example, using the nitrogen content of 0.3 gm. of powder of *Hypomyces ipomoeae* as a standard, 0.36 gm. of powder of *F. javanicum* had to be used per set of injections to supply an equivalent amount of nitrogen. (As a reciprocal control, a pair of animals received 0.245 gm. of powder of *H. ipomoeae* per series of injections. This amount was approximately equal in nitrogen content to 0.3 gm. of powder of *F. javanicum* or *F. theobromae*.) Similarly, using the 1:50 stock extract of *H. ipomoeae* (0.3 gm. of powder in 15 cc. saline) as a standard, the nitrogen equivalent extract of *F. javanicum* was made from 0.37 gm. of powder in 15 cc. of saline. Results of this experiment are given in table VI.

The chemical analysis and serological tests do not reveal identity of the organisms as one would expect. In fact, the culture designated *F. javanicum* so far as nitrogen content and serological reaction are concerned stands closer to the culture designated *H. ipomoeae* than the culture designated *F. theobromae*.

It was found that the antisera of animals receiving inject antigens supposedly containing the same amounts of nitrogen showed differences in titer equal to those observed in animals immunized with the standard amounts of powders. Similarly, when test antigens sup-

posedly containing equivalent amounts of nitrogen were used in comparative tests with standard test antigens, the same magnitude and orders of difference in titer were noted in both.

The conclusion was drawn that quantitative difference in nitrogen content of inject and test antigens is not a critical factor in explaining the non-specific results obtained.

TABLE VI

IMMUNE SERA $I_2$

| Organism | Hypomyces ipomoeae | | | | F. theobromae | | | | F. javanicum | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.3 gm.* | | 0.245 gm.* | | 0.3 gm.* | | 0.37 gm.* | | 0.3 gm.* | | 0.36 gm.* | |
| | Titer | Sum | Titer | Sum | Titer | Sum | Titer | Sum | Titer | Sum | Titer | Sum |
| Hypomyces ipomoeae (0.3)† | 6400 | 175 | 3200 | 115 | 3200 | 185 | 6400 | 185 | 6400 | 155 | 3200 | 145 |
| Hypomyces ipomoeae (0.3)† | 6400 | 170 | 3200 | 140 | 6400 | 185 | 6400 | 180 | 6400 | 175 | 3200 | 135 |
| F. theobromae (0.3)† | 1600 | 65 | 800 | 60 | 1600 | 140 | 1600 | 105 | 1600 | 75 | 1600 | 75 |
| F. theobromae (0.307) | 1600 | 60 | 800 | 60 | 3200 | 100 | 1600 | 105 | 1600 | 70 | 1600 | 70 |
| F. javanicum (0.3)† | 3200 | 160 | 1600 | 105 | 6400 | 180 | 3200 | 145 | 6400 | 135 | 1600 | 95 |
| F. javanicum (0.37) | 6400 | 145 | 1600 | 90 | 6400 | 160 | 3200 | 130 | 3200 | 140 | 1600 | 105 |
| F. fructigenum (0.3)† | 3200 | 95 | 1600 | 85 | 6400 | 145 | 1600 | 120 | 3200 | 125 | 800 | 55 |
| F. fructigenum (0.27) | 1600 | 70 | 1600 | 70 | 6400 | 175 | 1600 | 120 | 3200 | 115 | 800 | 60 |
| S. sclerotiorum (0.3)† | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S. sclerotiorum (0.825)‡ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* This figure represents number of grams of powder received by each animal per series of injections.

† This figure represents grams of powder used in 1:50 stock test antigen dilution.

‡ This figure represents grams of powder used in 1:50 stock test antigen dilution, one-half the N equivalent amount.

4. PRECIPITIN ABSORPTION TESTS.—This series of experiments was undertaken in an attempt to determine whether or not absorption of precipitins would afford differentiation of organisms where the precipitin test failed to do so.

Fragmentary tests were run with *F. cepae* vs. *F. oxysporum*, *F. fructigenum* vs. *F. callistephi*, *F. javanicum* vs. *Hypomyces ipomoeae*, *F. nivale* vs. *F. argillaceum*, and *Gibb. saubinetii* no. 259 vs. *Gibb. saubinetii* (English strain). Fuller tests were run with *F. conglutinans* var. *callistephi*, *F. cubense* no. 8, and *F. lycopersici*, reciprocally. Detailed intensive tests were run with *F. conglutinans* vs. *F. lycopersici*,

*Neurospora tetrasperma* (single ascospore strain A) vs. *N. tetrasperma* (single ascospore strain B), (two lots of antigen), and *S. sclerotiorum* vs. two strains of *S. fructicola*.

Although in the section on methods some suggestions as to approximate amounts of extract or powder and serum, and periods and types of incubation were given, a routine method of precipitin absorption has not yet been found. The suggestions indicate a possible range of partial or complete absorption within which the desired conditions might be attained.

For every serum-antigen combination used there was absorption. In some cases this was complete, the absorbed serum giving no reaction against the antigen with which it had been absorbed; in others absorption was partial, as indicated by the diminished titer of the serum and appropriate antigen; or serum was overabsorbed, as indicated by anomalous reactions. There were a few absorption tests among the many run in which proper absorption occurred. In the detailed studies on the *Sclerotinia* species, results obtained from partial absorption checked those obtained in complete absorption. In the detailed studies on the *Neurospora* strains, partial absorption results checked complete absorption results with very few exceptions. (For a second lot of powder grown in each case, these absorptions were all incomplete and therefore inconclusive.) Detailed studies on *F. conglutinans* and *F. lycopersici* showed that partial absorptions checked complete absorption except that all absorption results with serum obtained shortly after immunization were of one type while all of those with serum obtained some weeks after immunization were of a different type.

The absorptions obtained did indicate the possession by each organism of a specific antigenic fraction as well as a common antirenic fraction shared with the other organism. Examples are *N. tetraspermum* A vs. *N. tetraspermum* B, *S. sclerotiorum* vs. *S. fructicola* nos. 1 and 2, and *S. fructicola* no. 1 vs. *S. fructicola* no. 2, data for which are given in tables VII–IX.

On the whole, results obtained with absorption of precipitins were inconclusive because of the unreliability of the technique used. Thus a method giving complete absorption at one time might fail to do so when the tests were repeated using a sample of the same serum and

different antigen lots, or the same antigen and different serum lots. Nevertheless the data indicate that the precipitin absorption meth-

TABLE VII

| Organism | Unabsorbed sera | | | Absorbed sera | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $F_1$ | $F_2$ | S | $F_1/F_1$† | $F_1/F_2$ | $F_1/S$ | $F_2/F_1$ | $F_2/F_2$ | $F_2/S$ | $S/F_1$ | $S/F_2$ | S/S |
| S. fructicola #1 ($F_1$)... | 400* | 800 | 200 | 0 C‡ | 100 P | 200 S | 0 C | 0 C | 200 N | 0 C | 0 C | 0 C |
| S. fructicola #2 ($F_2$)... | 400 | 400 | 200 | 50 P | 40 P | 400 S | 40 P | 0 C | 200 S | 40 P | 0 C | 100 P |
| S. sclerotiorum (S).... | 800 | 800 | 3200 | 0 C | 0 C | 0 C | 0 C | 0 C | 0 C | 800 S | 800 S | 200 P |

TABLE VIII

| Organism | Unabsorbed sera | | Absorbed sera | | | |
|---|---|---|---|---|---|---|
| | $F_1$ | $F_2$ | $F_1/F_1$† | $F_1/F_2$ | $F_2/F_2$ | $F_2/F_1$ |
| S. fructicola #1 ($F_1$)..... | 200* | 200 | 0 C‡ | 40 P | 0 C | 0 C |
| S. fructicola #2 ($F_2$)..... | 100 | 200 | 0 C | 40 ±? | 0 0 | 50 P |

TABLE IX

| Organism | Unabsorbed sera | | Absorbed sera | | | |
|---|---|---|---|---|---|---|
| | A | B | A/A | A/B | B/B | B/A |
| N. tetrasperma A (A)..... | 1600* | 800 | 0 C‡ | 30 P | 0 C | 0 C |
| N. tetrasperma B (B)..... | 1600 | 1600 | 0 C | 0 C | 0 C | 30 P |

* Numerals represent titers.

† In $F_1/F_1$, etc., numerator represents serum; denominator, absorbing antigen.

‡ C, complete absorption; P, partial absorption; S, very slight absorption; N, no absorption of homologous precipitin by heterologous organism.

od has possibilities, and when adequately controlled it may be an alternative means of differentiation in cases in which the precipitin test fails.

These supplementary tests indicate that variations due to age and history of antisera, variable hydrogen-ion concentration of test antigens, and variable nitrogen contents of powders and extracts are not individual limiting factors which might account for the non-specific reactions obtained. The results with absorption of precipitins indicate that the presence of common antigenic and haptenic substances may be responsible for some of the non-specific precipitations. Perfection of this technique gives promise of sharper differentiations.

Further possibilities that might account for the non-specific reactions obtained are: (1) the specific fractions of the organism may not be extractable with 0.85% NaCl solution (such as certain polysaccharides and lipoids); (2) the extract may be too diverse chemically. Extracts of greater chemical homogeneity, such as the purified globulins prepared from cell contents by NELSON (**12**), and from the different constituents of the hyphal walls, may make differentiation possible. In the present study specific reactions may have been masked in part by reactions of common cell wall constituents; (3) absorption of precipitins on spores and hyphal fragments may be more serviceable than such absorptions with extracts and powders.

## VI. Summary

1. Potent antisera and test antigens (giving maximum titers of 1:25,600) were prepared from 34 species and strains of fungi by using fractions soluble in 0.85% NaCl solution.

2. The fractions of some organisms exhibited sufficient specificity to permit differentiation, but in most cases cross precipitin reactions were so strong that identification was impossible. In a few cases absorption of precipitins differentiated fungi not separable by the precipitin test.

3. Thus in a limited number of reciprocal tests, members of the Pezizales (*Sclerotinia* species) were differentiated from members of the Hypocreales (*Neurospora tetrasperma*, *Fusarium* species, and a species each of *Cylindrocarpon* and *Ramularia*), certain ones of which were in turn distinguished from one another. Strong group reactions occurred among all members of the genus *Fusarium* tested. The precipitin test did not permit as sharp differentiation between the

monilioid strains of *N. tetrasperma* and the *Fusarium* group as it did between *Fusarium* species and the conidial stages of *Cylindrocarpon album* and a *Ramularia* sp.

4. Two genera of the Pezizales (*S. sclerotiorum* and *S. fructicola*) and two strains of *S. fructicola*, as well as the plus and minus strains of *N. tetrasperma*, were differentiated by means of the precipitin absorption test.

5. Many attempts to demonstrate specific precipitabilities for the saline extracts of certain cultures recognized as strains and species of *Fusarium* (some of which are recognized as conidial stages of different genera of Ascomycetes) were unsuccessful.

6. At present, therefore, the procedure described in this paper is not invariably usable for identification of every one of the fungi tested, although consideration of all reactions of every organism leads to the conclusion that each is a distinct serological entity.

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# EFFECTS OF TEMPERATURE ON METABOLISM IN TOMATO[1]

G. T. NIGHTINGALE

(WITH TWO FIGURES)

## Introduction

In the practice of agriculture, it has always been recognized that seasonal climatic changes exert a dominant influence on plant growth, and for years this was considered to be due mainly to temperature. In 1920, GARNER and ALLARD (**10**) demonstrated that plant response was definitely affected by the seasonal length of day or photoperiod. It has also been shown that photoperiodic influences or effects may be materially modified by nutrient treatment (**18**). Obviously, plants are affected by all factors of the environment to which they are subjected. It is the purpose of this paper to point out certain effects of temperature.

## Experimental methods

Plants of the tomato (*Lycopersicon esculentum* Mill.) were employed for these experiments, the Bonny Best variety being selected. The plants were propagated from seed and grown in 4-inch clay pots in sifted loam soil until they had reached a height of about 8 cm. At that time the roots were washed free of foreign material and set

[1] Through the courtesy of the University of Chicago there was most generously made available for these experiments the temperature-humidity control equipment and laboratory facilities of its Department of Botany. Some of the results thus obtained are reported in the following pages. Other papers will appear subsequently in this journal; and in the Contributions from Boyce Thompson Institute will be published the results of the work of ECKERSON (**9**), who made microchemical analyses, anatomical observations, and reducase determinations on the plants used for these experiments.

Kjeldahl and mineral determinations were made in the laboratory of C. S. CATHCART, for whose cooperation the writer wishes to express appreciation. He is also indebted to G. B. ULVIN, who assisted in making macro-determinations of chlorophyll. Special acknowledgment is due C. H. HARRISON, who for the two months preceding the actual temperature experiments grew the plants in sand culture, giving daily attention to nutrient applications and producing plants of the quality of growth desired for the experiments.

in washed quartz sand in new, washed, 10-inch clay pots, five plants to a pot. The pots were then set in shallow enamelware pans, and until the time of temperature treatments all of the plants received daily applications of minus-N nutrient solution (table I) in sufficient quantity to flush the sand and fill the pans. If the solution in the pans became low between periods of nutrient application, distilled water was added, although this was not often necessary. During the period preceding the time when they were subjected to the experimental temperature treatments, the plants were grown under usual greenhouse conditions with a temperature of 70°–75° F. during the day and of 65°–70° F. during the night.

TABLE I

COMPOSITION OF NUTRIENT SOLUTION (PARTIAL VOLUME MOLECULAR CONCENTRATIONS OF SALTS USED)

| | $Ca(NO_3)_2$ | $KH_2PO_4$ | $MgSO_4$ | $CaCl_2$ |
|---|---|---|---|---|
| Complete or plus-$NO_3$ | 0.0090 | 0.0045 | 0.0045 | ..... |
| Minus-N | ..... | 0.0045 | 0.0045 | 0.0090 |

On April 26, 1932, after about six weeks of minus-N treatment, some of the plants (hereafter referred to as *initial* plants) were harvested for chemical analysis and the remainder were placed in the glass inclosed chambers designed for control of temperature and humidity. The glass of these chambers and of the greenhouse roof materially reduced light intensity as compared with that in the open. Air in the chambers was changed continuously and rapidly. It was obtained from outside the greenhouse and contained no artificially added carbon dioxide. These factors in relation to the results obtained will be discussed elsewhere. Three chambers were employed, with a relative humidity of 85 per cent in all of them, but the temperatures were 55°, 70°, and 95° F. respectively (13°, 21°, and 35° C.). Sudden changes in intensity of sunlight resulted in temporary fluctuations in temperature. In any case, however, the temperature did not vary more than $\pm 1.5°$ from the standard set, and then for only a few minutes at any given time. Humidity was also controlled with about the same degree of accuracy.

Each temperature chamber accommodated 20 pots, or 100 plants. For a period of 19 hours all the plants continued to receive the minus-N nutrient treatment at the respective temperatures indicated. At the end of that time half the plants at each temperature were given the complete or plus-$NO_3$ nutrient solution (table I), and the remainder continued to receive the solution lacking nitrogen. These nutrient solutions were in all cases thereafter applied daily in the manner already described.

The plants were divided for analysis into fibrous roots, whole stems, blades, and petioles, the last portion including also the rachis and large veins. Only the results of macro-analyses of stems are reported in detail, however; the other parts of the plant followed very closely in trend the changes which occurred in the stem.

Determinations of nitrogenous and carbohydrate fractions were made with fresh and dried tissues respectively, according to procedure previously described in detail (**20, 21**). Aliquots of dried tissue were employed for mineral analyses (**1**).

Chlorophyll was extracted from representative aliquots of fresh blades according to the method of SCHERTZ (**31**) and the amount estimated colorimetrically using GUTHRIE'S (**11**) reagent as a standard.

## Results

### INITIAL PLANTS

The initial plants, as well as those used for experimental treatments, were selected from a large population and were uniform in size, quality, and appearance. On April 26, after six weeks of minus-N treatment, they were, as might be anticipated, typical nitrogen-deficient plants (fig. 1). The stems were about 25 cm. long but stiff and woody. The base of the stem was purplish blue to light yellow. The upper two or three leaves were fairly dark green but the lower ones were distinctly yellow, with purple veins. There were practically no blossoms present. The root system was unusually large in proportion to the tops and consisted of many fine white extensive roots.

The plants were high in carbohydrates but very low in all forms of organic nitrogen, and contained neither nitrate nor ammonium (tables II–V). Cell walls of mechanical and conductive tissue were

FIG. 1.—Experiments started on April 26, 1931: On left, typical *initial* tomato plants representative of those employed for experimental treatments. The three sets of plants on right are as they appeared on May 2, after receiving nutrient solution lacking nitrogen and the temperature treatments indicated (plants at 95° F. were considerably taller and more spindling at the time of final harvest on May 5).

thick, and starch was observed in large quantities in all parenchymatous tissue nearly to the tip of the stem (9).

Macro-analyses for minerals which are not reported in detail showed that the initial plants contained an abundance of phosphorus, potassium, calcium, magnesium, and sulphur.

ECKERSON (9) found that the leaves, especially of the initial plants, were very high in nitrate-reducing material. The plants were in every respect in ideal condition for protein synthesis and rapid growth, if given an available nitrate supply and favorable conditions of environment.

## RESPONSES OF PLANTS AT 70° F.

1. PLANTS DEFICIENT IN NITRATES.—These plants were harvested for analysis on May 5, ten days after being subjected to a continuous temperature of 70° F. During that period there was comparatively little change in the appearance (fig. 1) and chemical composition (tables II–VI) of the plants as compared with their initial condition on April 26. There was, however, a slight increase in volume. They appeared darker green and there was increase in chlorophyll (table VI). A similar response but in greater degree was exhibited by the minus-N 95° plants.

2. PLANTS WHICH RECEIVED THE COMPLETE NUTRIENT SOLUTION. —Nitrate absorption was apparently instantaneous (9) and nitrate was found in all parts of the plant about four hours following application of the complete nutrient solution. Absorption of nitrate was accompanied by rapid reduction to nitrite, ammonium, and amino acid synthesis (9). Thirty-six hours after the plants received nitrate the leaves were visibly darker green. When finally harvested for analysis on May 5, the plants had increased greatly in volume (fig. 2), the new stem growth and new leaves were soft and succulent, and all parts of the plant were dark green and growing vigorously. No blossoms formed on this nor on any other group of plants during the ten-day period of temperature treatments.

Accompanying vigorous growth there was a marked decrease in concentration of carbohydrates (tables II, III) and increase in organic nitrogen (tables IV, V), and with change from yellowish to

FIG. 2.—Experiments started on April 26, 1931: On left, typical *initial* tomato plants representative of those employed for experimental treatments. The three sets of plants on right are as they appeared on May 2, after receiving complete nutrient solution and the temperature treatments indicated (at time of final harvest on May 5, the plants at 70° F. had increased at least a third more in volume and those subjected to 95° had increased in height, but the new growth was extremely soft and spindling).

dark green the percentage chlorophyll content of the blades more than doubled (table VI).

TABLE II

CARBOHYDRATE FRACTIONS OF WHOLE STEMS EXPRESSED AS PERCENTAGE OF DRY MATTER, AND DRY MATTER EXPRESSED AS PERCENTAGE OF GREEN MATTER

| | Percentage | | | | | | |
|---|---|---|---|---|---|---|---|
| | April 26 | May 5 | | | May 5 | | |
| | Initial plants | Minus-N | | | Plus-$NO_3$ | | |
| | | 55° F. | 70° F. | 95° F. | 55° F. | 70° F. | 95° F. |
| Dry matter | 14.00 | 14.60 | 13.60 | 12.60 | 12.20 | 8.60 | 10.00 |
| Reducing sugars | 11.57 | 11.57 | 8.60 | 11.15 | 7.29 | 6.80 | 7.15 |
| Sucrose | 3.42 | 8.98 | 7.53 | 3.58 | 4.85 | 2.47 | 2.74 |
| Total sugars | 14.99 | 20.55 | 16.13 | 14.73 | 12.14 | 9.27 | 9.89 |
| Starch and dextrin | 17.33 | 22.15 | 24.05 | 7.87 | 16.60 | 9.88 | 2.55 |
| Total carbohydrates | 32.32 | 42.70 | 40.18 | 22.60 | 28.74 | 19.15 | 12.44 |

TABLE III

CARBOHYDRATE FRACTIONS OF WHOLE STEMS EXPRESSED AS PERCENTAGE OF GREEN MATTER

| | Percentage | | | | | | |
|---|---|---|---|---|---|---|---|
| | April 26 | May 5 | | | May 5 | | |
| | Initial plants | Minus-N | | | Plus-$NO_3$ | | |
| | | 55° F. | 70° F. | 95° F. | 55° F. | 70° F. | 95° F. |
| Reducing sugars | 1.62 | 1.69 | 1.17 | 1.40 | 0.89 | 0.58 | 0.72 |
| Sucrose | 0.48 | 1.31 | 1.02 | 0.45 | 0.59 | 0.21 | 0.27 |
| Total sugars | 2.10 | 3.00 | 2.19 | 1.85 | 1.48 | 0.79 | 0.99 |
| Starch and dextrin | 2.43 | 3.23 | 3.27 | 0.99 | 2.03 | 0.85 | 0.26 |
| Total carbohydrates | 4.53 | 6.23 | 5.46 | 2.84 | 3.51 | 1.64 | 1.25 |

TABLE IV

NITROGENOUS FRACTIONS OF WHOLE STEMS EXPRESSED AS PERCENTAGE OF DRY MATTER

| | Percentage | | | | | | |
|---|---|---|---|---|---|---|---|
| | April 26 | May 5 | | | May 5 | | |
| | Initial plants | Minus-N | | | Plus-$NO_3$ | | |
| | | 55° F. | 70° F. | 95° F. | 55° F. | 70° F. | 95° F. |
| Total nitrate-free N | 0.670 | 0.490 | 0.655 | 0.770 | 0.806 | 1.211 | 0.808 |
| Protein N | 0.453 | 0.266 | 0.405 | 0.394 | 0.494 | 0.393 | 0.377 |
| Nitrate-free soluble N | 0.217 | 0.224 | 0.250 | 0.376 | 0.312 | 0.818 | 0.431 |
| Basic N | 0.077 | 0.060 | 0.083 | 0.041 | 0.079 | 0.268 | 0.050 |
| Amino N | 0.150 | 0.110 | 0.169 | 0.225 | 0.127 | 0.475 | 0.136 |
| Amide N | 0.007 | 0.020 | 0.014 | 0.033 | 0.084 | 0.053 | 0.181 |
| Other N | −0.017 | 0.034 | −0.016 | 0.077 | 0.022 | 0.022 | 0.064 |
| Nitrate N | None | None | None | None | 0.404 | 0.519 | 0.622 |
| Total N | 0.670 | 0.490 | 0.655 | 0.770 | 1.270 | 1.730 | 1.430 |

TABLE V

NITROGENOUS FRACTIONS OF WHOLE STEMS EXPRESSED AS PERCENTAGE OF GREEN MATTER

| | Percentage | | | | | | |
|---|---|---|---|---|---|---|---|
| | April 26 | May 5 | | | May 5 | | |
| | Initial plants | Minus-N | | | Plus-$NO_3$ | | |
| | | 55° F. | 70° F. | 95° F. | 55° F. | 70° F. | 95° F. |
| Total nitrate-free N | 0.093 | 0.072 | 0.089 | 0.097 | 0.098 | 0.104 | 0.081 |
| Protein N | 0.063 | 0.039 | 0.055 | 0.050 | 0.060 | 0.034 | 0.038 |
| Nitrate-free soluble N | 0.030 | 0.033 | 0.034 | 0.047 | 0.038 | 0.070 | 0.043 |
| Basic N | 0.011 | 0.009 | 0.011 | 0.005 | 0.010 | 0.023 | 0.005 |
| Amino N | 0.021 | 0.016 | 0.023 | 0.028 | 0.015 | 0.041 | 0.014 |
| Amide N | 0.001 | 0.003 | 0.002 | 0.004 | 0.010 | 0.004 | 0.018 |
| Other N | −0.003 | 0.005 | −0.002 | 0.010 | 0.003 | 0.002 | 0.006 |
| Nitrate N | None | None | None | None | 0.057 | 0.045 | 0.062 |
| Total N | 0.093 | 0.072 | 0.089 | 0.097 | 0.155 | 0.149 | 0.143 |

### Responses of plants at 55° F.

1. Plants deficient in nitrates.—During the ten-day period at 55° F. the plants with continuous minus-N nutrient treatment did not noticeably increase in volume (fig. 1) but definitely increased in concentration of carbohydrates (tables II, III). There being no external nitrogen supply, there necessarily occurred a decrease in percentage of nitrogen, especially as expressed on a dry weight basis (table IV). Microscopic examination (9) confirmed these results, as shown by the fact that starch was present even in the extreme tip of the stem.

TABLE VI

Chlorophyll in leaf blades of tomato

| | April 26 | May 5 | | | May 5 | | |
|---|---|---|---|---|---|---|---|
| | | Minus-Nitrogen | | | Plus-Nitrate | | |
| | Initial plants | 55° F. | 70° F. | 95° F. | 55° F. | 70° F. | 95° F. |
| As percentage of green matter... | 0.048 | 0.033 | 0.054 | 0.048 | 0.039 | 0.099 | 0.078 |
| Relative values*. | 48 | 33 | 55 | 48 | 39 | 100 | 79 |

* Calculated using the plus-nitrate 70° F. plants, on May 5, as 100.

During the low temperature treatment the leaves became lighter yellow, and there was loss of chlorophyll (table VI) and apparently destruction of chloroplasts (9). The stems and veins, however, exhibited a very heavy purplish red anthocyanin pigmentation.

2. Plants which received the complete nutrient solution.—Nitrate absorption was apparently instantaneous (9), and in about five hours nitrates were present in all parts of the plant. Translocation seemed therefore to be a little slower than at 70° F. The 55° plants both with and without nitrates gradually decreased in capacity for nitrate reduction (9), although, as may be seen from a comparison of figures 1 and 2, addition of nitrates materially increased the leaf area and increased slightly the height of the plants. Growth with nitrates at 55°, however, was extremely slow. At 70° there was a noticeable response after 36 hours, but only after four or five days at 55° F.

The increase in leaf area of the plus-$NO_3$, 55° plants was not accompanied by an increase in green color. On the contrary there was loss in concentration of chlorophyll (table VI) and disorganization of chloroplasts, although small and poorly developed chloroplasts appeared in the small apical leaves. These plants, like the minus-N treated individuals, had stiff woody stems, were yellow and heavily tinged with purple.

Associated with limited growth and nitrate assimilation there was, after ten days of 55° treatment, a much lower concentration of carbohydrates than in the minus-N plants at the same temperature (tables II, III).

Organic nitrogen was also higher, especially on a dry weight basis (table IV), and with increase in volume (fig. 2) there must have been increase in total organic nitrogen or there would otherwise have been a loss in percentage as expressed on a green weight basis. That this did not occur is indicated in table V and by the fact that on a green weight basis total organic nitrogen in the roots, stems, petioles, and blades was respectively 0.132, 0.098, 0.097, and 0.281 per cent at the time of final harvest on May 5, whereas corresponding figures for the *initial* plants on April 26 were 0.126, 0.093, 0.058, and 0.266.

### Responses of plants at 95° F.

1. Plants deficient in nitrates.—In less than eight hours after the plants had been subjected to a temperature of 95° F., the petioles, except those of the two lower leaves, became nearly vertical in position and formed an acute angle with the stem axis. This is clearly shown in figure 1. (At 55° and 70° the petioles were more nearly at right angles to the stem and almost horizontal in position.)

During the ten-day period at 95° there was a marked decrease in carbohydrates (tables II, III). In fact, microscopic examination (9), only 36 hours after shifting to the 95° chamber, showed that the amount of starch had materially decreased in the upper 4 cm. of stem tissue. Accompanying the decrease in carbohydrates there was a marked increase in concentration of total and soluble organic nitrogen, as expressed on a dry weight basis (table IV).

The 95° plants, even with no external nitrogen supply, increased noticeably in volume (fig. 1). On a green weight basis, therefore,

there was no increase in concentration of nitrogen (table V). It is significant, however, that without added nitrogen, and associated with increase in volume of the plant, there occurred a distinct decrease in protein and corresponding increase in soluble organic nitrogen (tables IV, V), especially in *other*, amino, and amide nitrogen.

After three or four days at 95°, accompanying the proteolytic response already recorded, the plants turned somewhat darker green. This was soon followed by injury to the chloroplasts (9), however, and when harvested for analysis on May 5, almost the entire plant was yellowish, practically free of anthocyanin pigments, and as low in chlorophyll as the initial plants (table VI).

2. PLANTS WHICH RECEIVED THE COMPLETE NUTRIENT SOLUTION. —It is apparent from figure 2 that these plants responded similarly to the minus-N treated plants at the same temperature with respect to the acute angle arrangement of the petiole and stem. This response became apparent in both groups of plants at about the same time. Further discussion of this point will be found in a paper by ECKERSON (9).

Application of nitrates was followed immediately by their absorption, and nitrate was found in all parts of the plant in a little less than four hours (9) after application. Nitrate reduction (9) also was extremely rapid during the first few hours at 95° F. After that period reducase activity apparently gradually dropped, until on May 5 (after ten days) there was practically no reducase (9) in either plus-$NO_3$ or minus-N treated plants. This response was clearly reflected externally. In less than 24 hours after nitrate application, the foliage was distinctly darker green than that of any other groups of plants in these experiments; and for three or four days there was a continued increase in stem height and leaf area that far exceeded in rate the response of the plus-$NO_3$ plants at 70° F. This rapid growth of the plants in the 95° chamber was correlated with decrease in carbohydrate content, especially in the upper half of the plants, and with increase in amino acids and asparagine (9).

After four or five days at 95°, however, the growth rate became relatively slow. As may be seen from tables II and III, there was an extreme decline in carbohydrates. These data, however, are for whole stems. The upper halves of the plants (9) were practically

devoid of starch on May 5. Also on that date a few of the plants (which were not employed for analysis) were dead at the stem tips. The upper leaves, which were very dark green during the first few days, were also rapidly turning yellow. At the time of final harvest the chlorophyll content of aliquots of blades was lower than that found in the plus-$NO_3$ group at 70° (table VI).

At the time of analysis on May 5, the stems of the plus-$NO_3$, 95° plants were surprisingly low in organic nitrogen (tables IV, V). The petioles, however, contained on a green weight basis about four times the percentage of organic nitrogen found on April 26 in the same part of the initial plants, and the blades had about doubled in concentration of nitrate-free nitrogen during that period.

Following the ten-day experimental period and the final harvest for analysis, a few plants were kept in the chamber at 95° F. All those receiving complete nutrient died within two or three days. This was in striking contrast to the minus-N treated plants, which showed no indication of death of stem tips nor of other parts during the same period.

During the ten-day period in the temperature chambers the external appearance of the root systems of the respective series was much the same, and the rate of growth corresponded closely to that of the tops. Toward the end of the period of treatment at 95°, however, there was definite injury to the protoplasm of the fine roots (9); but no injury was apparent externally and the roots did not die until several days following the termination of these experiments.

## Discussion

### Significance of results

The plants of these experiments were subjected to practically constant temperature conditions, and humidity was maintained at approximately 85 per cent in each chamber.[2] Sunlight and carbon dioxide supply were variable but at any given time were essentially the same for all the temperature treatments. The responses of the plants at 55°, 70°, and 95° F. are therefore strictly comparable.

[2] Although the relative humidity was maintained at 85 per cent, the vapor pressure deficit of course varied at each of the three temperatures. Effects of relative humidity and of vapor pressure deficit will be discussed in a paper soon to appear.

Under the same conditions of nutrient treatment, differences observed in growth and metabolism were brought about solely as the result of differences in temperature, although obviously the response of the plants was influenced by all factors of their environment. It should be emphasized, however, that the respective temperatures imposed were continuous night and day. During the night, plants are commonly subjected to temperatures ranging lower than during the day. No attempt was made to duplicate such conditions.

Before discussing these results it would seem pertinent to recall certain relationships between temperature, carbon dioxide, and light. At the highest temperature employed (95° F.), the comparatively low light intensity of the glass inclosed chambers was undoubtedly a limiting factor in carbon dioxide assimilation (**14, 32**). Yet if more light had been supplied in an attempt to obtain maximal assimilation at 95°, it would also have been necessary to increase materially the concentration of atmospheric carbon dioxide (**14, 32**). Even if this had been accomplished, however, the factor of increased "inactivation" (**14, 32**) of the plant at higher temperatures would still have been impossible of elimination, as at 95° there was (table VI) definite injury to protoplasm and especially to chloroplasts (**9**).

## Carbohydrates

The process of protein synthesis from nitrates necessarily involves utilization of carbohydrates resulting in a decrease of those in storage, unless the amount is supplied by new synthesis. But if tomato plants already containing protein reserves are grown with no external nitrogen supply, carbohydrates in the plant will increase, provided the rate of carbon dioxide assimilation exceeds that of respiration, or will decrease if respiration predominates over photosynthesis.

At 55° with minus-N treatment there was a definite increase in carbohydrates in the stems (tables II, III) over that found in the initial plants. A corresponding increase occurred in all other parts of the plant (**9**), showing beyond question that carbohydrates were synthesized at 55° in greater quantity than they were respired. This occurred even though there was some loss in chlorophyll (table VI) and injury to chloroplasts (**9**). Likewise at 70° with no external nitrogen supply there was a gain in concentration of carbohydrates

in all parts of the plant but not so great as at 55° F. The somewhat lower carbohydrate content of the series at 70° is not surprising. It has already been pointed out that light and carbon dioxide were limiting factors to maximal assimilation at 70°, whereas respiration was undoubtedly accelerated by the higher temperature without serious limitation by any factor (**14, 32**). At 95° with minus-N treatment, it is clearly apparent (tables II, III) that there was a much greater loss of carbohydrates through respiration than was compensated for by photosynthesis.

No work seems to have been done by others on effects of temperature on the carbohydrate content of plants grown with no external nitrogen supply. The results of these experiments show beyond question, however, that the lower temperature of 55° resulted in carbohydrate accumulation in the tomato plant; whereas at 95° there was a decrease in carbohydrates. It is emphasized also that these effects of temperature were not the result of prolonged treatments. The total duration of the entire temperature treatment was only ten days in each case, and noticeable changes in carbohydrate content (**9**) occurred within a few hours after shifting the plants to the respective temperatures.

The proportion of carbohydrate fractions as determined (tables II, III) varied somewhat, yet the results (**15, 20**) seem to indicate as usual that in the tomato, starch is an important storage form, and that it accumulated with increase in other carbohydrates. That starch is not stored in all species of plants will be pointed out elsewhere.

## Reutilization of protein

The plants grown with no external nitrogen supply furnished information on carbohydrate metabolism that could not have been obtained had they been utilizing carbohydrates in the synthesis of proteins from nitrate. These plants are also of additional interpretative value in that any effects of temperature on nitrogen metabolism are limited strictly to effects on organic nitrogen already present in the plant. The situation is not complicated by nitrate assimilation and the consequent presence of newly synthesized nitrogenous compounds.

Figure 1 shows that the plants at 95° with no added nitrogen in the nutrient solution increased materially in volume as compared with the initial plants and with the plants at 55° F. They likewise turned darker green, although later, with injury to chloroplasts (**9**), and became yellowish. Associated with this growth there must obviously have been reutilization of organic nitrogen. Tables IV and V show this to be the case, since there was a definite increase in the simpler, more mobile forms of organic nitrogen as amino, amide, and *other* nitrogen, and a corresponding decrease in proteins and basic nitrogen, the latter fraction consisting partly of polypeptides.

The record of this response is not new (**18, 20, 21**), nor is it in any sense peculiarly related to high temperature alone. A nitrogen deficient tomato plant high in carbohydrate reserves, with yellowish green foliage, may be made to grow and turn dark green if the concentration of its contained carbohydrates is reduced and the percentage of its organic nitrogen thereby relatively increased, by subjecting the plant to shading or to a short photoperiod (**18**). That the high temperature plants of these experiments did decrease in carbohydrates to a great degree has already been indicated (tables II, III).

With the decreased light intensity of the temperature chambers, there was in the plants lacking nitrate at 70° some evidence of growth in the stem tips and terminal leaves. In these organs, however, carbohydrates were much lower (**9**) than is indicated by the average carbohydrate content of whole stems of the same plants, as determined macrochemically (tables II, III). At 55° the plants lacking nitrates made no noticeable growth as compared with those of the initial series, even though there was a considerable increase in concentration of amide and *other* nitrogen. This increase, however, may have been one of the results accompanying injury of the protoplasm and of the chloroplasts (**9**) at 55° (table VI), rather than proteolysis similar to that occurring at 70° and 95° F. Increase in amide and other nitrogen seems often to occur under somewhat comparable conditions (**3, 4, 18, 20**).

### Absorption and translocation of nitrate

Absorption of nitrate was not a limiting factor at any of the temperatures employed (**9**). As nearly as could be determined, there

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appeared to be immediate absorption of nitrate by the root hairs. On the basis of nitrate appearance within the roots, no difference in permeability could be detected at temperatures of 55°, 70°, and 95° F. Different results might have been obtained with more extreme temperatures (**7, 26, 36**). The rate of translocation through the root system and top was a little slower at 55° than at 70°, however, and in turn nitrates were found in all parts of the plants at 95° a little sooner than at 70° (**9**). Yet five hours after nitrates were added to the sand in which the plants were growing, there was an abundance of nitrate in all parts of the plants, even at 55°; and when harvested for analysis on May 5, the plants which were subjected to the lowest temperature had on a green weight basis a higher concentration of nitrate than the plants at 70° (tables IV, V). In the case of asparagus (**19**), it has been pointed out that at a temperature of 45° F. the absorption of nitrate was not limited but the same temperature inhibited its assimilation.

The plants at 55° made little growth (fig. 2), and as will be shown presently, used or assimilated little nitrate. In about five hours, therefore, they became filled with nitrate to maximum capacity and removed very little more from the culture medium, as was evidenced by no tendency toward pH change (**12, 13**) of the residual nutrient solution. On the basis of nitrate removed from the culture solution, it might be said that at 55° temperature limited absorption; yet obviously such an interpretation is inaccurate, especially if it implies that the root system is impermeable to nitrate.

In connection with absorption of nitrate, plants of the various temperature and nutrient treatments were analyzed for calcium, magnesium, potassium, phosphorus, and sulphur. The plants without nitrate had almost exactly the same concentration of each element at 55°, 70°, and 95° F. Likewise the nitrate supplied plants at 55°, which were assimilating little nitrate, were only slightly higher. All of these elements (**8, 16, 21, 23**), however, including sulphur in certain forms (**22**), are known to be required in abundance in rapidly growing tissues. It seems logical, therefore, that there was found in the vigorously growing plants with complete nutrient at 70° a considerably higher percentage of each of these elements than was present in comparatively inactive plants. The plants which for a time

were making vigorous growth at 95° with nitrate, were at the end of ten days only a little lower in these elements than the nitrate supplied plants at 70° F.

### Nitrate assimilation

It has been indicated that absorption and translocation of nitrate were not limiting factors at any of the temperatures employed in these experiments. Nitrates were present in high concentration in all parts of the plants (**9**) at each temperature. The high percentage of nitrate nitrogen in the stems is reported in tables III and IV. Nitrates in themselves, however, will not produce growth (**18, 20**). They are not an essential part of the living protoplasm of plants, but rather represent an excess of nutrient material not yet metabolized.

On the basis of growth response alone, it is clearly apparent from figure 2 that there was, at the respective temperatures employed, a marked difference in ability to utilize nitrate. The greatest growth was made at 70°, and in these plants Eckerson (**9**) found a consistently high concentration of nitrate reducing material. Likewise in this group of plants, accompanying rapid synthesis of amino acids (**9**), there necessarily occurred oxidation or decrease in carbohydrates (tables II, III). Associated with these changes there was, in the first 48 hours after the initial addition of nitrate to the nutrient medium, noticeable growth of the plant as a whole and change in color of foliage from yellowish to darker green, until finally on May 5, ten days later, the plants had approximately doubled the chlorophyll content of the blades (table VI) and had become materially lower in percentage of carbohydrates but comparatively high in content of all determined forms of organic nitrogen. Correlated with these internal changes the plants grew vigorously as usual (**15, 20, 21**), and the new growth was comparatively soft and succulent.

The plants at 55° decreased rapidly in content of nitrate reducing material, until finally at the end of ten days reducase was practically absent (**9**). It is apparent (fig. 2), however, that added nitrate at 55° resulted in increased growth, although growth and assimilation of nitrate were extremely slow as compared with that at 70° F. At the lower temperature there was scarcely any perceptible growth during the first five days, and after this period the rate of growth

was even slower. Associated with this great retardation in growth there was little, if any, nitrate assimilation. Such limited assimilation of nitrate at 55° is further indicated by the somewhat higher concentration of organic nitrogen in the stems (tables IV, V) and other plant parts of those supplied with nitrate as compared with the initial plants and comparable plants lacking nitrate. The notably high concentration of amide, however, may not have been entirely a synthetic product (**3, 4, 20**).

Accompanying limited assimilation of nitrate at 55° there necessarily occurred some decrease in reserve carbohydrate content, and the nitrate supplied plants at this temperature, although remaining extremely high in carbohydrates, showed a definitely lower concentration of sugars and starch (tables II, III) than the initial plants or the series without nitrate at the same temperature.

There was also exhibited externally the usual symptoms (**15, 20**) characteristic of plants high in carbohydrates but deficient in proteins. Growth was slow and stems were hard, woody, and heavily tinged with the bluish purple of anthocyanins; the foliage was conspicuously yellow, probably partly because of protein deficiency but also because of gradual (**9**) decrease in chlorophyll (table VI) under the low temperature treatment.

In connection with these responses, the work of ROSA (**29**) is of particular interest. He grew tomato plants in soil in a "warm greenhouse" and others in a cold-frame where they were "exposed to temperatures near freezing during the night and to full sunlight during the day." The plants in the cold-frame apparently responded much as did those just described. He made no nitrogen determinations, but found that plants in the cold-frame were more than three times as high in starch as those in the warm greenhouse. In cabbage, ROSA found that similar treatment resulted in accumulation of carbohydrates, these consisting of sugars and pentosans rather than of starch.

There seem to be few references which deal directly with the effects of temperature on nitrate assimilation, although WALSTER (**34**) found that carbohydrates accumulated in much higher concentrations in barley plants that were grown in a greenhouse at approximately 15° C. than was the case in plants subjected to a greenhouse

temperature of about 20° C. He ran nitrogen analyses, but only of the tops, which leaves doubt as to the interpretation of his results, since in the Gramineae it has been reported (**30**) that nitrate reduction occurs mainly in the roots. In the asparagus plant also nitrate assimilation is limited almost exclusively to the fine roots, and assimilation of nitrate in these organs (**19**) apparently does not occur at temperatures below 10° C.

Effects of temperature will, of course, vary with the species and variety of plants employed. Yet experiments are consistent in showing that moderately low temperature results in such typical responses as hastened or accentuated tuberization in potato (**2**); premature seeding in plants such as beet (**5**), cabbage (**29**), cauliflower (**28**), and celery (**27**); and earlier grain development of barley (**34**). Such responses seem typically[3] to occur (**15, 20**) when plants are accumulating carbohydrates, not when there is rapid assimilation of nitrate and consequent decrease in carbohydrates. Decreased nitrate elaboration, however, is only one reason for increase in concentration of carbohydrates. It will be recalled that tomato plants at 55° with no nitrate supply gained materially in percentage and amount of sugars and starch, apparently because photosynthetic activity at that temperature predominated over respiration.

There is little pertinent information available on effects of higher temperatures on the growth and composition of plants. In connection with the responses of tomato at 95°, it may again be pointed out that under the conditions of these experiments the rate of respiration exceeded carbohydrate synthesis. During the early stages of growth

[3] PLATENIUS (**27**) demonstrated conclusively that low temperature hastened seeding in celery, but he did not find consistent differences in composition of seeders and non-seeders. He analyzed whole plants on the assumption that "all parts undergo similar changes in composition under each treatment." Such an assumption is obviously not tenable, and too, vegetative and reproductive plants have very different proportions of blade, petiole, and storage root tissue; and in a given plant each of these parts is extremely different in composition. Kind or quality of growth seems (**15, 20**) to be intimately associated with quality of or percentage composition. The percentage composition of a heterogeneous whole-plant aliquot may be of doubtful significance, and absolute amounts per whole plant are correlated with plant size, not quality.

Starch is only one (**6**) of the many non-nitrogenous storage reserves in plants; and although PLATENIUS analyzed for sugars as well as for starch, he made no determinations of mannite, which is notably high in celery (**24, 25, 35**).

at 95°, when the reserve carbohydrate content of the plants was still high (tables II, III), nitrate reduction (**9**) and growth were more vigorous and rapid than at 70°; but after the higher temperature had been imposed upon the plants for ten days, their reducase content had dropped to practically nothing (**9**). As they synthesized organic nitrogen, the nitrate supplied plants of course lost carbohydrates (tables II, III) more rapidly than those with no nitrate.

At the end of the ten-day period occasional plants of the complete nutrient series at 95° were dead at the tips (and were discarded). Examination of other plants from this series showed that in the upper halves of the tops carbohydrates were extremely low (**9**), and had decreased very greatly throughout the stems as a whole (tables II, III). The plants obviously lost chlorophyll, as analyses ultimately indicated (table VI). In the latest stages of growth the plants were weakly vegetative, and very soft and succulent, a condition that frequently (**15, 18, 20, 21**) accompanies extreme deficiency in utilizable carbohydrates.

At the time of final harvest for analysis, the stems (tables IV, V) were not high in organic nitrogen; but, as already mentioned, apparently there was translocation of organic nitrogen to other organs of the plant, principally to the petioles. In both stems and petioles there was associated with carbohydrate deficiency the usual (**3, 4, 18, 20**) marked decrease in percentage of protein and increase in the less complex fractions, especially amide and *other* nitrogen (tables IV, V).

## Summary

Tomato plants were grown in sand culture in glass inclosed chambers and humidity was maintained at 85 per cent in all cases. Sunlight and carbon dioxide supply varied but at any given time were the same for each temperature treatment. The following relative *differences* which occurred in growth and metabolism at 55°, 70°, and 95° F. were brought about as the result of differences in temperature.

1. TEMPERATURE 55° F. (13° C.).—At this temperature carbohydrates, especially starch, accumulated in large quantities, indicating beyond question that assimilation of carbon dioxide exceeded respiration.

Nitrate was absorbed instantaneously, and in about five hours

was present in high concentration throughout the plant and remained high.

Nitrate was assimilated (synthesized to organic nitrogen) very slowly and the plants were very low in nitrate reducing material (9). Accordingly, carbohydrates were high even in nitrate supplied plants, as there was little utilization of carbohydrates in protein synthesis.

Externally the plants were yellowish green, owing to a low chlorophyll content and comparatively few and poorly developed chloroplasts. The veins and stems were purplish, from the presence of anthocyanins. The stems were hard and woody and the cell walls thick. This was true even of plants supplied with nitrate, although these conditions were accentuated in plants lacking nitrate.

2. TEMPERATURE 70° F. (21° C.).—Plants lacking an external nitrogen supply were not so high in carbohydrates as comparable plants at 55° F., and exhibited symptoms of protein deficiency in less degree.

Nitrate absorption was apparently instantaneous and translocation of nitrate was slightly more rapid than at 55° F.

The plants were high in nitrate reducing material (9) and assimilation of nitrate was rapid, hence there was a comparatively high concentration of carbohydrates in plants lacking nitrate as compared with those receiving nitrate.

Plants supplied with a complete nutrient solution were moderately high in carbohydrates, contained an abundance of elaborated nitrogen, were dark green, rather succulent, and grew vigorously.

3. TEMPERATURE 95° F. (35° C.).—Plants with no external nitrogen supply as well as those with complete nutrient rapidly decreased in carbohydrates, indicating that respiration exceeded carbon dioxide assimilation at this temperature.[4]

Accompanying decrease in concentrations of carbohydrates, there was a breaking down of complex proteins to simpler forms of organic nitrogen, accelerated growth for a few days, and death of the plants.

Nitrate absorption was apparently instantaneous and translocation of nitrate was a little more rapid than at 55° or 70° F.

[4] With increase in temperature, limiting effects of carbon dioxide supply and light intensity are accentuated, page 47.

At first nitrate assimilation and reducase activity (9) were extremely high and the external response occurred much sooner than at 70°, but after four or five days nitrate assimilation practically ceased and the nitrate supplied plants grew much more slowly than at 70° F.

Because of carbohydrate utilization in protein synthesis, as well as in respiration, the plants which received nitrate lost carbohydrates more rapidly and died sooner than those with no external nitrogen supply.

During the early stages of 95° treatment, the plants receiving a complete nutrient solution grew rapidly and were dark green, although spindling. After a few days there was disintegration of chloroplasts, the newly developed leaves contained few and small chloroplasts (9), and the plants as a whole were soft, yellowish green, mottled, and practically free of anthocyanin pigmentation.

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# A CLASSIFICATION OF THE ALGAE BASED ON EVOLUTIONARY DEVELOPMENT, WITH SPECIAL REFERENCE TO PIGMENTATION

JOSEPHINE E. TILDEN

(WITH PLATE I)

## Introduction

In early treatises on algae these plants were grouped according to color; later this was considered to be an artificial method. It now seems, however, that the pigments characteristic of the various types must have been of the most vital importance in the development and advancement of these organisms; therefore a classification with pigmentation as a basis is entirely logical. Accordingly such names for the classes of algae as have reference to color should be retained and a new name adopted to include the several types of algae in which the chromatophore has a golden yellow, greenish yellow, or yellowish brown hue. PASCHER'S (**35**) term "Chrysophyceae" seems very appropriate, unless it should be proved in the future that the yellowish color is due in all cases to an excess of xanthophyll, when it would seem better to substitute the name "Xanthophyceae," recently suggested by ALLORGE (**1**) to take the place of "Heterocontae."

The special pigmentation character belonging to each group of algae must be a heritable character of high permanence; evidently it was definitely established in the organism during its early history, for certain types of the five races of algae are today found growing side by side under identical environments.

The writer (TILDEN **41, 42**) believes that the primary factor in the differentiation of these groups was a gradual increase in the quantity, or change in the quality, through incalculable periods of time, of solar energy, which reached the organism after its passage through the two overlying media, air and water. The individual pigments and groups of pigments present in the cells of living algae may be compared to index fossils in beds of sediment. They were succes-

sively developed in response to an environment of gradually changing radiant energy, and they furnish in themselves infallible evidence of sequence in time.

It is the purpose of this paper to call attention to the fact that, whatever may be our conception of the prototype of algal organisms, the working basis for new investigation in this important group of plants lies in the data we have at hand concerning the plants which are in existence today. From these facts alone we must make our interpretation of conditions in the past and develop a plan for attacking the problems of the future. Toward this end, a new method of arranging and classifying the mass of details with which all students of algae are familiar is herewith submitted, in the hope that it may be of value in indicating new lines of research.

The table presented in plate I embodies the views of the writer with regard to the evolution of the algae. Algal "periods" have been inserted in the geological time scale, and corresponding to these periods is shown the group of pigments characteristic of each class of algae. It would seem safe to place the beginning of the algae in general in the Archaeozoic. The position of each class indicates its possible or probable point of origin. It must be understood that types of living algae only are included.

## Phylogenetic relationships as indicated by cytological evidences, pigments, and food reserves

In building up a classification of the algae, it seems logical to begin with the group of living forms presenting the simplest type of cell structure.

### Cyanophyceae

The work of Prat (**38**), Mockridge (**30**), and Poljansky and Petruschewsky (**37**) has made it clear that the Cyanophycean cell possesses no organized nucleus. Poljansky and Petruschewsky emphasize the following points in connection with their investigations on species of *Spirulina*, *Oscillatoria*, *Tolypothrix*, and *Gloeotrichia*. The cell is made up of (1) a cellular membrane; (2) a peripheral plasma consisting of protoplasm diffusely saturated with pigments; and (3) a central body (the central colorless portion of the cell) which contains metachromatic granules, stored assimilation products such as glycogen, and a "chromatic substance."

POLJANSKY and PETRUSCHEWSKY do not hold the view that the peripheral plasma should be considered as a limited chromatophore, since the central body is not sharply outlined but seems to change gradually into the peripheral plasma. Nor, in their opinion, is it correct to conclude that the central colorless portion of the cell is a nucleus without a membrane. It is more nearly correct to compare the basal substance of the central body with the cytoplasm of the ordinary cell. On the other hand, there is a substance in the central mass which definitely gives the nuclear reaction and stains with basic dye. The form in which this substance occurs varies from one species to another, appearing as a network, a spongy mass, or as strands stretched out in a longitudinal direction. To this chromatin-like material they have applied the name "chromatic substance." There is no reason to assume that the definite parallel arrangement of the chromatic material, observed during division in some species, is a primitive mitosis. The division of a cell of *Spirulina*, for example, may be characterized as a single constriction of the contents of the cell into two parts, during which process there is developed a new cellular membrane. The chromatic substance forms no chromosomes during its division, but divides by simple fission. The chromatic substance, however, is chemically either identical with, or closely related to, the chromatin of a true nucleus. Supposedly these structures are also phylogenetically homologous.

Cyanophycin granules are particularly abundant in hypnogonidia. At least in the case of *Gloeotrichia*, they are to be considered as inclusion bodies of the peripheral plasma.

Thus a definite picture of the form and structure of a simple cell, or unicellular plant, is gained from the conclusions of POLJANSKY and PETRUSCHEWSKY. The same picture may represent an ideal ancestral organism, for the simplest living algal plants of today (that is, unless reduced or retrograde) probably resemble the ancestral form far more closely than do any of the higher algae.

PIGMENTATION.—It is assumed that the progenitors of the blue-green algae came into being during the period of weakest illumination, the "Cyanophycean period."

Most of the species belonging to the Cyanophyceae are pale, dull, or vivid bluish green in color; a few are rose-violet or brown. These

various tints are due to the presence of certain pigments in the protoplast, of which *phycocyanin*, a blue pigment, is the dominant one. *Phycoerythrin*, a rose-red or violet-red pigment, is more or less abundant in certain species, but it is believed to be entirely lacking in others. Chlorophyll and carotinoid pigments are present, but practically nothing is known of the "chlorophyll-carotinoid" complex of the blue-green algae. No one has been able to establish the chemical formula for any one of their pigments.

Phycocyanin alone, or in association with phycoerythrin, may have as its function the absorbing of the penetrating rays of light and the transferring of them to the chlorophyll and carotinoid, as the centers of the process of photosynthesis. DANILOV (**10, 11**) investigated the water-soluble pigments of blue-green algae grown in pure culture, and also of red algae collected in the Arctic Ocean. He doubts the protein nature of the pigments and thinks it more probable that they are dissolved in the proteins. KLUGH (**20**) suggests that the pigment known as phycoerythrin is an optical sensitizer which enables the chlorophyll of the algae possessing it to make use of the radiations which would, without its presence, be extremely ineffective. On the other hand, phycocyanin and phycoerythrin themselves may be as much concerned in photosynthesis as is the chlorophyll-carotinoid combination.

With reference to the presence or absence of the pigments phycocyanin and phycoerythrin, the Cyanophyceae fall into three divisions according to GAIDUKOV (**14**), BOCAT (**3**), KYLIN (**24, 26**), BORESCH (**5, 6**), ISHIKAWA (**18**), WILLE (**45**), and NAKANO and HIGASHI (**31**). They are as follows:

1. *Blue-green algae containing phycocyanin alone.*—The majority of the blue-green algae contain phycocyanin only. Ordinarily they exhibit a blue-green or Spanish green color:

Oscillatoria tenuis, O. formosa
Phormidium corium, Ph. laminosum var. aeruginea,
Ph. autumnale var. aeruginea
Anabaena sp.

2. *Blue-green algae containing both phycocyanin and phycoerythrin.*—These forms are blue-green or dark olive-green in color:

Oscillatoria sancta, O. okeni, O. limosa, O. amoena, O. caldariorum
Phormidium favosum, Ph. persicinum, Ph. subfuscum, Ph. laminosum var. olivacea-fusca, Ph. autumnale var. olivacea, Ph. retzii var. nigro-violacea
Lyngbya aerugineo-caerulea
Microchaete calotrichoides
Phylloderma sacrum

WILLE (45) made some interesting observations on *Phormidium persicinum*, which he found on the south coast of Norway. His notes, written in 1889, bear out recent findings with respect to these pigments. At a depth of 18 feet an abundance of *Laminaria saccharina* occurred on which were growing some Bryozoan colonies. The empty shells of the Bryozoa contained large numbers of *Ph. persicinum* of a Floridean (Rhodophycean) red color. With the help of the Zeiss absorption spectroscope, he compared the blue-green alga with a red one, *Rhodymenia palmata*, and states that the absorption spectra of *Ph. persicinum* and *R. palmata* are specific, and therefore that *Ph. persicinum* contains genuine phycoerythrin. WILLE concludes that, together with other conditions, this points toward the phylogenetic relation of the Florideae (Rhodophyceae) to the Cyanophyceae. The phycocyanin-containing Cyanophyceae occur only near the surface, while *Ph. persicinum* grows at depths of 18–24 feet or more, where usually only red algae thrive. The substitution of phycoerythrin for phycocyanin at greater depths is apparently connected with the difference in their property of absorbing light.

3. *Blue-green algae containing phycoerythrin alone (or with very little phycocyanin)*.—These are the dark olive-brown, light sepia-brown, brownish red, and brownish violet varieties:

Oscillatoria cortiana
Phormidium luridum and its two varieties, fusca and violacea
Microchaete tenera

FOOD RESERVES IN CYANOPHYCEAE.—Glycogen in the blue-green algae appears to function much as starch does in the higher green

plants. The studies of POLJANSKY and PETRUSCHEWSKY reveal several interesting facts concerning this substance. Apparently the presence of glycogen is connected with the physiological condition of the alga. It occurs usually in the peripheral plasma. Only a small amount was determined in species of *Gloeotrichia* and *Tolypothrix*, while *Oscillatoria* and *Spirulina* contained a much greater quantity. In *Oscillatoria princeps* it was found to be present in both the central and the peripheral portions of the protoplast.

### RHODOPHYCEAE

A long period of time must have elapsed between the Cyanophycean and Rhodophycean periods, in which in turn the blue-green and the red algae constituted the dominant flora of the sea. All that remains today to indicate the transitional forms are a few species of red algae usually designated as the Protoflorideae. The period of Rhodophyceae must have been characterized by a light intensity somewhat corresponding to that at a depth in sea water which the majority of the red algae seem to prefer.

It seems not unlikely that, in some of the mutants of the ancient blue-green algal cell, the scattered masses of chromatic material might have gained in size; that eventually the separate particles might have taken on a rodlike shape; and that gradually these rod-shaped bits might have been collected into a small portion of the cell within a drop of more concentrated and finely granular protoplasm. Such a structure would approach that of a primitive nucleus.

In the Protoflorideae there is a distinct nucleus. ISHIKAWA's (**18**) cytological studies on *Porphyra tenera*, however, convince one that he was working with a simple type of nucleus. He considered that the mode of nuclear division might be taken as a primitive mitosis, midway between amitosis and mitosis. In addition to this nucleus ISHIKAWA distinguished a distinct chromatophore. A peculiar pyrenoid appears in the cell and likewise the stored carbohydrate, known as "Floridean starch."

The fresh-water unicellular form so common in greenhouses, *Porphyridium cruentum*, until recently was included under the Cyanophyceae; but a nucleus being discovered in the cell as well as a star-shaped chromatophore, it was transferred to the Protoflorideae.

In all of the members of the Rhodophyceae other than this small group, the nucleus of the cell appears to be of as high a type as that of other plants and animals.

PIGMENTATION.—As in the supposed development of a nucleus, a similar gathering together of the separate pigment particles into a slightly differentiated mass of protoplasm would constitute a simple chromatophore. It has already been shown that there must have been a gradual transition in the phycocyanin-phycoerythrin pigments of the blue-green algae, since we still have the descendants of those algae containing phycocyanin alone, phycocyanin associated with phycoerythrin, and phycoerythrin alone.

Whether the chlorophyll of the red algae is in the form of *a* and *b* has not yet been ascertained, but both carotin and xanthophyll are reported by HANSEN (**17**), KYLIN (**24, 25, 26**), PALMER (**34**), and others. Phycoerythrin, the red pigment, is so abundant that as a general rule it entirely masks the other pigments. Phycocyanin is found to be present in some cases and absent in others, according to KYLIN (**24, 26**), ISHIKAWA (**18**), and WILLE (**45**).

1. *Red algae containing phycocyanin alone.*—In a very small number of species phycocyanin is present and phycoerythrin absent:

Asterocytis ramosa

Batrachospermum vagum, B. testale, B. virgatum

2. *Red algae containing both phycoerythrin and phycocyanin.*—Both pigments are found in numerous species of red algae (KYLIN):

Protoflorideae:

Bangia fusco-purpurea

Porphyra tenera, P. hiemalis, P. umbilicalis

Euflorideae:

Freshwater forms:

Batrachospermum dillenii, B. gallaei, B. moniliforme, B. helminthosum

Lemanea fluviatilis

Marine forms:

Nemalion multifidum

Dumontia filiformis

Furcellaria fastigiata

Ahnfeltia plicata

At first nitrate assimilation and reducase activity (9) were extremely high and the external response occurred much sooner than at 70°, but after four or five days nitrate assimilation practically ceased and the nitrate supplied plants grew much more slowly than at 70° F.

Because of carbohydrate utilization in protein synthesis, as well as in respiration, the plants which received nitrate lost carbohydrates more rapidly and died sooner than those with no external nitrogen supply.

During the early stages of 95° treatment, the plants receiving a complete nutrient solution grew rapidly and were dark green, although spindling. After a few days there was disintegration of chloroplasts, the newly developed leaves contained few and small chloroplasts (9), and the plants as a whole were soft, yellowish green, mottled, and practically free of anthocyanin pigmentation.

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# A CLASSIFICATION OF THE ALGAE BASED ON EVOLUTIONARY DEVELOPMENT, WITH SPECIAL REFERENCE TO PIGMENTATION

JOSEPHINE E. TILDEN

(WITH PLATE I)

## Introduction

In early treatises on algae these plants were grouped according to color; later this was considered to be an artificial method. It now seems, however, that the pigments characteristic of the various types must have been of the most vital importance in the development and advancement of these organisms; therefore a classification with pigmentation as a basis is entirely logical. Accordingly such names for the classes of algae as have reference to color should be retained and a new name adopted to include the several types of algae in which the chromatophore has a golden yellow, greenish yellow, or yellowish brown hue. PASCHER's (**35**) term "Chrysophyceae" seems very appropriate, unless it should be proved in the future that the yellowish color is due in all cases to an excess of xanthophyll, when it would seem better to substitute the name "Xanthophyceae," recently suggested by ALLORGE (**1**) to take the place of "Heterocontae."

The special pigmentation character belonging to each group of algae must be a heritable character of high permanence; evidently it was definitely established in the organism during its early history, for certain types of the five races of algae are today found growing side by side under identical environments.

The writer (TILDEN **41, 42**) believes that the primary factor in the differentiation of these groups was a gradual increase in the quantity, or change in the quality, through incalculable periods of time, of solar energy, which reached the organism after its passage through the two overlying media, air and water. The individual pigments and groups of pigments present in the cells of living algae may be compared to index fossils in beds of sediment. They were succes-

sively developed in response to an environment of gradually changing radiant energy, and they furnish in themselves infallible evidence of sequence in time.

It is the purpose of this paper to call attention to the fact that, whatever may be our conception of the prototype of algal organisms, the working basis for new investigation in this important group of plants lies in the data we have at hand concerning the plants which are in existence today. From these facts alone we must make our interpretation of conditions in the past and develop a plan for attacking the problems of the future. Toward this end, a new method of arranging and classifying the mass of details with which all students of algae are familiar is herewith submitted, in the hope that it may be of value in indicating new lines of research.

The table presented in plate I embodies the views of the writer with regard to the evolution of the algae. Algal "periods" have been inserted in the geological time scale, and corresponding to these periods is shown the group of pigments characteristic of each class of algae. It would seem safe to place the beginning of the algae in general in the Archaeozoic. The position of each class indicates its possible or probable point of origin. It must be understood that types of living algae only are included.

## Phylogenetic relationships as indicated by cytological evidences, pigments, and food reserves

In building up a classification of the algae, it seems logical to begin with the group of living forms presenting the simplest type of cell structure.

### Cyanophyceae

The work of Prat (**38**), Mockridge (**30**), and Poljansky and Petruschewsky (**37**) has made it clear that the Cyanophycean cell possesses no organized nucleus. Poljansky and Petruschewsky emphasize the following points in connection with their investigations on species of *Spirulina*, *Oscillatoria*, *Tolypothrix*, and *Gloeotrichia*. The cell is made up of (1) a cellular membrane; (2) a peripheral plasma consisting of protoplasm diffusely saturated with pigments; and (3) a central body (the central colorless portion of the cell) which contains metachromatic granules, stored assimilation products such as glycogen, and a "chromatic substance."

POLJANSKY and PETRUSCHEWSKY do not hold the view that the peripheral plasma should be considered as a limited chromatophore, since the central body is not sharply outlined but seems to change gradually into the peripheral plasma. Nor, in their opinion, is it correct to conclude that the central colorless portion of the cell is a nucleus without a membrane. It is more nearly correct to compare the basal substance of the central body with the cytoplasm of the ordinary cell. On the other hand, there is a substance in the central mass which definitely gives the nuclear reaction and stains with basic dye. The form in which this substance occurs varies from one species to another, appearing as a network, a spongy mass, or as strands stretched out in a longitudinal direction. To this chromatin-like material they have applied the name "chromatic substance." There is no reason to assume that the definite parallel arrangement of the chromatic material, observed during division in some species, is a primitive mitosis. The division of a cell of *Spirulina*, for example, may be characterized as a single constriction of the contents of the cell into two parts, during which process there is developed a new cellular membrane. The chromatic substance forms no chromosomes during its division, but divides by simple fission. The chromatic substance, however, is chemically either identical with, or closely related to, the chromatin of a true nucleus. Supposedly these structures are also phylogenetically homologous.

Cyanophycin granules are particularly abundant in hypnogonidia. At least in the case of *Gloeotrichia*, they are to be considered as inclusion bodies of the peripheral plasma.

Thus a definite picture of the form and structure of a simple cell, or unicellular plant, is gained from the conclusions of POLJANSKY and PETRUSCHEWSKY. The same picture may represent an ideal ancestral organism, for the simplest living algal plants of today (that is, unless reduced or retrograde) probably resemble the ancestral form far more closely than do any of the higher algae.

PIGMENTATION.—It is assumed that the progenitors of the blue-green algae came into being during the period of weakest illumination, the "Cyanophycean period."

Most of the species belonging to the Cyanophyceae are pale, dull, or vivid bluish green in color; a few are rose-violet or brown. These

various tints are due to the presence of certain pigments in the protoplast, of which *phycocyanin*, a blue pigment, is the dominant one. *Phycoerythrin*, a rose-red or violet-red pigment, is more or less abundant in certain species, but it is believed to be entirely lacking in others. Chlorophyll and carotinoid pigments are present, but practically nothing is known of the "chlorophyll-carotinoid" complex of the blue-green algae. No one has been able to establish the chemical formula for any one of their pigments.

Phycocyanin alone, or in association with phycoerythrin, may have as its function the absorbing of the penetrating rays of light and the transferring of them to the chlorophyll and carotinoid, as the centers of the process of photosynthesis. DANILOV (**10, 11**) investigated the water-soluble pigments of blue-green algae grown in pure culture, and also of red algae collected in the Arctic Ocean. He doubts the protein nature of the pigments and thinks it more probable that they are dissolved in the proteins. KLUGH (**20**) suggests that the pigment known as phycoerythrin is an optical sensitizer which enables the chlorophyll of the algae possessing it to make use of the radiations which would, without its presence, be extremely ineffective. On the other hand, phycocyanin and phycoerythrin themselves may be as much concerned in photosynthesis as is the chlorophyll-carotinoid combination.

With reference to the presence or absence of the pigments phycocyanin and phycoerythrin, the Cyanophyceae fall into three divisions according to GAIDUKOV (**14**), BOCAT (**3**), KYLIN (**24, 26**), BORESCH (**5, 6**), ISHIKAWA (**18**), WILLE (**45**), and NAKANO and HIGASHI (**31**). They are as follows:

1. *Blue-green algae containing phycocyanin alone.*—The majority of the blue-green algae contain phycocyanin only. Ordinarily they exhibit a blue-green or Spanish green color:

Oscillatoria tenuis, O. formosa
Phormidium corium, Ph. laminosum var. aeruginea,
Ph. autumnale var. aeruginea
Anabaena sp.

2. *Blue-green algae containing both phycocyanin and phycoerythrin.*—These forms are blue-green or dark olive-green in color:

Oscillatoria sancta, O. okeni, O. limosa, O. amoena, O. caldariorum
Phormidium favosum, Ph. persicinum, Ph. subfuscum, Ph. laminosum var. olivacea-fusca, Ph. autumnale var. olivacea, Ph. retzii var. nigro-violacea
Lyngbya aerugineo-caerulea
Microchaete calotrichoides
Phylloderma sacrum

WILLE (45) made some interesting observations on *Phormidium persicinum*, which he found on the south coast of Norway. His notes, written in 1889, bear out recent findings with respect to these pigments. At a depth of 18 feet an abundance of *Laminaria saccharina* occurred on which were growing some Bryozoan colonies. The empty shells of the Bryozoa contained large numbers of *Ph. persicinum* of a Floridean (Rhodophycean) red color. With the help of the Zeiss absorption spectroscope, he compared the blue-green alga with a red one, *Rhodymenia palmata*, and states that the absorption spectra of *Ph. persicinum* and *R. palmata* are specific, and therefore that *Ph. persicinum* contains genuine phycoerythrin. WILLE concludes that, together with other conditions, this points toward the phylogenetic relation of the Florideae (Rhodophyceae) to the Cyanophyceae. The phycocyanin-containing Cyanophyceae occur only near the surface, while *Ph. persicinum* grows at depths of 18–24 feet or more, where usually only red algae thrive. The substitution of phycoerythrin for phycocyanin at greater depths is apparently connected with the difference in their property of absorbing light.

3. *Blue-green algae containing phycoerythrin alone (or with very little phycocyanin)*.—These are the dark olive-brown, light sepia-brown, brownish red, and brownish violet varieties:

Oscillatoria cortiana
Phormidium luridum and its two varieties, fusca and violacea
Microchaete tenera

FOOD RESERVES IN CYANOPHYCEAE.—Glycogen in the blue-green algae appears to function much as starch does in the higher green

plants. The studies of POLJANSKY and PETRUSCHEWSKY reveal several interesting facts concerning this substance. Apparently the presence of glycogen is connected with the physiological condition of the alga. It occurs usually in the peripheral plasma. Only a small amount was determined in species of *Gloeotrichia* and *Tolypothrix*, while *Oscillatoria* and *Spirulina* contained a much greater quantity. In *Oscillatoria princeps* it was found to be present in both the central and the peripheral portions of the protoplast.

### RHODOPHYCEAE

A long period of time must have elapsed between the Cyanophycean and Rhodophycean periods, in which in turn the blue-green and the red algae constituted the dominant flora of the sea. All that remains today to indicate the transitional forms are a few species of red algae usually designated as the Protoflorideae. The period of Rhodophyceae must have been characterized by a light intensity somewhat corresponding to that at a depth in sea water which the majority of the red algae seem to prefer.

It seems not unlikely that, in some of the mutants of the ancient blue-green algal cell, the scattered masses of chromatic material might have gained in size; that eventually the separate particles might have taken on a rodlike shape; and that gradually these rod-shaped bits might have been collected into a small portion of the cell within a drop of more concentrated and finely granular protoplasm. Such a structure would approach that of a primitive nucleus.

In the Protoflorideae there is a distinct nucleus. ISHIKAWA'S (**18**) cytological studies on *Porphyra tenera*, however, convince one that he was working with a simple type of nucleus. He considered that the mode of nuclear division might be taken as a primitive mitosis, midway between amitosis and mitosis. In addition to this nucleus ISHIKAWA distinguished a distinct chromatophore. A peculiar pyrenoid appears in the cell and likewise the stored carbohydrate, known as "Floridean starch."

The fresh-water unicellular form so common in greenhouses, *Porphyridium cruentum*, until recently was included under the Cyanophyceae; but a nucleus being discovered in the cell as well as a star-shaped chromatophore, it was transferred to the Protoflorideae.

In all of the members of the Rhodophyceae other than this small group, the nucleus of the cell appears to be of as high a type as that of other plants and animals.

PIGMENTATION.—As in the supposed development of a nucleus, a similar gathering together of the separate pigment particles into a slightly differentiated mass of protoplasm would constitute a simple chromatophore. It has already been shown that there must have been a gradual transition in the phycocyanin-phycoerythrin pigments of the blue-green algae, since we still have the descendants of those algae containing phycocyanin alone, phycocyanin associated with phycoerythrin, and phycoerythrin alone.

Whether the chlorophyll of the red algae is in the form of *a* and *b* has not yet been ascertained, but both carotin and xanthophyll are reported by HANSEN (**17**), KYLIN (**24, 25, 26**), PALMER (**34**), and others. Phycoerythrin, the red pigment, is so abundant that as a general rule it entirely masks the other pigments. Phycocyanin is found to be present in some cases and absent in others, according to KYLIN (**24, 26**), ISHIKAWA (**18**), and WILLE (**45**).

1. *Red algae containing phycocyanin alone.*—In a very small number of species phycocyanin is present and phycoerythrin absent:

Asterocytis ramosa
Batrachospermum vagum, B. testale, B. virgatum

2. *Red algae containing both phycoerythrin and phycocyanin.*—Both pigments are found in numerous species of red algae (KYLIN):

Protoflorideae:
Bangia fusco-purpurea
Porphyra tenera, P. hiemalis, P. umbilicalis
Euflorideae:
Freshwater forms:
Batrachospermum dillenii, B. gallaei,
B. moniliforme, B. helminthosum
Lemanea fluviatilis
Marine forms:
Nemalion multifidum
Dumontia filiformis
Furcellaria fastigiata
Ahnfeltia plicata

Phyllophora membranacea
Chondrus crispus
Ceramium rubrum
Laurencia pinnatifida

3. *Red algae containing phycoerythrin alone.*—In the majority of red algae phycoerythrin is not accompanied by phycocyanin:

Protoflorideae:
Porphyridium cruentum
Eufloridеae

ISHIKAWA (**18**), working on the cytology of *Porphyra tenera*, did not hesitate to state that in his opinion the coexistence of the two sorts of coloring matter proves an intimate relation existing between *Porphyra* and the blue-green algae. He agrees with OKAMURA in believing that *Porphyra* has a close affinity with the Cyanophyceae, having a connecting link in *Porphyridium cruentum*. In general he feels that the evidence strongly favors the view that the Rhodophyceae sprang from a Cyanophycean stock, and that the higher Rhodophyceae (Eufloridеae) descended through the Bangiales (Protoflorideae).

FOOD RESERVES IN RHODOPHYCEAE.—KOLKWITZ (**21**) proved that "Floridean starch," a substance in the form of granules found in the cells of the Rhodophyceae, is mainly an assimilating product, or a reserve material. The granules stain brownish with iodine, but if in any way they are made to swell and are then treated with iodine, they show different colorations. KOLKWITZ found Floridean starch in all the red algae of the North Sea which he examined. He differentiates two types of starch: a *Laurencia* type with light wine-red color, and a *Furcellaria* type of bluish violet tones. In some red algae blue shades appear which are almost identical with the customary starch reaction, but the true shade was never obtained. In future investigations it may be found that Floridean starch consists of different amyloses, or related substances, occurring in various amounts in the different species, thus causing a different coloration with iodine in each case. While it is a fact that small molecules of ordinary starch give variable blue and red tints upon testing, the structure of Floridean starch undoubtedly deviates from that of true starch.

The granules of Floridean starch lie in the cytoplasm, but they begin their formation at the surface of the chromatophore as flat plates which become somewhat conical, the flattened bases remaining in contact with the plastid as long as they continue to grow. Apparently in some cases they are attached to the inner concave surface of the chromatophore. The older granules are free in the protoplasm. OLTMANNS (**33**) suggests their similarity to pyrenoid-like structures which may be formed without any connection with the chromatophores. CLELAND (**8**) reports a close association of the pyrenoid of *Nemalion* with the formation of Floridean starch. The findings therefore indicate that granules of some material which contains carbohydrates are present, and that this material constitutes the assimilation product of the Rhodophyceae.

Another substance which occasionally may be observed in the red algae (as in *Laurencia* and *Plocamium*, for example) is oil. No particular study has been made in this field, and therefore no additional facts are at hand.

## PHAEOPHYCEAE

It is assumed that new conditions of illumination were ushered in during the Phaeophycean period, and that possibly shallow seas, gradually sloping shores, and numerous tide-pools were in existence. Penetration of the radiance of the sun attained fuller power but by no means equaled that of the present time.

There are no transitional forms to make it possible to understand what happened to cause development of the new type of marine plants, other than the changes in solar energy. The brown seaweeds, as we know them, range from simple filaments to massive treelike structures.

PIGMENTATION.—Neither phycocyanin nor phycoerythrin occurs in the brown algae. The Phaeophycean algae living today have a peculiar olive-brown, olive-green, or brownish green color, and are characterized by a brown pigment which was announced by SORBY (**40**) in 1873. He proposed for it the name of "fucoxanthin."

WILLSTÄTTER and PAGE (**46**) completed the proof that chlorophyll is contained in the brown algae. WILLSTÄTTER and STOLL (**47**) state that the chlorophyll of these plants shows a remarkable deviation from that of land plants as well as from that of the green algae.

It consists almost exclusively of the *a* component, while only traces of chlorophyll *b* are to be observed, at most 5 per cent. They state: "The chlorophyll component *a*, isolated from the Fucoideae by WILLSTÄTTER and PAGE, agrees, in its magnesium and phytol content and in the composition of the nitrogen-containing cleavage products, with the pigment from other plants."

The brown algae contain a much greater amount of yellow pigment than do the green algae and land plants. The chlorophyll is masked by them because the yellow pigments predominate quantitatively. "The molecular ratio of the green to the yellow pigments is here about 1:1 instead of 3 to 5:1 as in many land plants" (**47**).

TSWETT (**43**) proved that three carotinoids are present in these seaweeds: carotin, xanthophyll, and fucoxanthin. WILLSTÄTTER and PAGE determined the quantitative distribution of the carotinoids. Their formulas are given on plate I. Of the three nitrogen-free pigments, fucoxanthin is richer in oxygen than the other carotinoids, its formula being $C_{40}H_{54}O_6$.

FOOD RESERVES IN PHAEOPHYCEAE.—KYLIN (**27, 28**) suggested the name "fucosan" for certain vacuoles or vesicles found in the cells of brown algae, particularly in the assimilation tissue and in the reproductive organs. According to him these vesicles contain a substance related to the tannins.

In all brown algae mannite is present as a reserve material. OLTMANNS (**32**) remarked: "It is, therefore, possible that the supposed phloroglucin plays only a secondary part; and evidence is increasing that the assimilation products of the Brown Algae are carbohydrates." KYLIN has proved this to be a fact. He has found monosaccharides (dextrose and levulose) in the Laminarieae and the Fucaceae. These sugars, as in the higher plants, are soon transformed into polysaccharides and are demonstrable in small quantities.

In the Laminarieae, "laminarines," a group of polysaccharides, are found stored, but the individual sugars have not been investigated as yet. In *Laminaria saccharina* and *L. digitata*, laminarines form up to 35 per cent of the dried material by weight; *Fucus serratus*

contains 19 per cent; *F. vesiculosus* and *Ascophyllum* only 7 per cent. In the last species the carbohydrates are substituted in considerable quantities by fat. According to MANGENOT (**29**), oil is formed by phaeoplasts in some of the brown algae.

The metabolism of the assimilation material of the Phaeophyceae, therefore, is completed in very much the same manner as in the green plants. The monosaccharides produced have been proved by KYLIN to form disaccharides, which are changed into polysaccharides. This substance is not starch, however.

### CHRYSOPHYCEAE

In the Chrysophycean period there apparently came into existence great numbers of new types, most of them unicellular, motile during at least a part of their life cycle, microscopic in size, and far more simple in structure than any of the forms immediately preceding them. The present day representatives of this group indeed manifest a strong resemblance to the reproductive bodies of the Phaeophycean algae, and, when ciliated (as they are in most of the subclasses) they exhibit the laterally inserted, unequal cilia characterizing that class. They contain a distinct nucleus, one or more brownish or yellowish chromatophores, and usually one to several pulsating vacuoles.

PIGMENTATION.—In the classification introduced in the present paper, a number of different groups (WEST and FRITSCH **44**) have been placed as subclasses under the general class name Chrysophyceae. The algae belonging to these several subclasses are in many cases very abundant at the present time, some in salt water, some in fresh water, and many in both habitats. They all agree in having golden brown or yellowish green chromatophores. Apparently the chlorophyll-carotinoid combination remains, fucoxanthin has disappeared, while an excess of xanthophyll gives to the chromatophore the characteristic golden tint. Since most of the forms are unicellular, the study of their pigments has so far been difficult or impossible, but it is probably safe to state that the Chrysophycean forms now living are characterized by a chromatophore possessing an excess of xanthophyll.

PIGMENTATION AND FOOD RESERVES IN THE VARIOUS SUBCLASSES OF THE CHRYSOPHYCEAE.—

TRIBONEMEAE

This group includes filamentous, unicellular, and coenocytic algae. The cells contain one, two, several, or many chromatophores, usually devoid of pyrenoids. The color is a peculiar yellow-green and is due to an excess of xanthophyll. Such chromatophores characterize all the Tribonemeae and are invariably associated with an absence of starch.

These plants, without exception, build through photosynthesis oil or fatlike droplets which are assumed to be formed from carbohydrates.

DIATOMEAE

The diatoms possess various kinds of chromatophores: small and discoid, large and platelike, band-shaped, and lobed. They are usually yellow or golden brown in color, very rarely green. While they contain the usual types of pigments, there is a preponderance of a brown substance which may or may not be xanthophyll (BEIJERINCK 2).

The products of photosynthesis accumulate as drops of fatty oil which are usually conspicuous in the cells under favorable conditions of nutrition. During rapid growth the oil decreases in amount, but increases again as growth becomes less active. The oil is usually observed outside the chromatophores, but it has also been found within them.

PERIDINEAE

Dark yellow or brown chromatophores, usually numerous and discoid, are characteristic of these algae.

Reserve food is stored as oil and in some cases as starch.

CRYPTOMONADINEAE

As a rule there are two large brown parietal chromatophores, mostly apposed to the dorsal and ventral margins of the organism. Rarely the chromatophores are discoid and numerous. Nothing is known about the nature of the pigments, but various shades in addition to brown are seen, as red, olive-green, and blue-green.

After active photosynthesis the products are stored as solid discoid granules which in some species seem to be a form of starch.

### Chrysomonadineae

This subclass is characterized by the possession of chromatophores of a golden yellow or brown color, but when the organisms are living in a habitat rich in organic substances, the chromatophores become greenish in tint.

The products of photosynthesis are stored as oil and leucosin. Starch is not known to occur. Leucosin has not been thoroughly investigated, but it is thought to be a carbohydrate. It occurs as colorless, highly refractive, usually rounded lumps (Pascher **36**).

### Chloromonadineae

The numerous discoid chromatophores exhibit a peculiar bright green tint which is due to the presence of xanthophyll in greater or less amount.

Food reserves are stored as oil.

### Euglenineae

The chromatophores exhibit a pure green color. They are discoid, lobed, or band-shaped, and there may be several or many in an organism.

In many species fat globules act as a food reserve. Starch is never demonstrable, but a peculiar substance, named paramylon by Gottlieb (**16**) because its composition resembled that of starch, is common. From tests it may be concluded that paramylon is a polysaccharide. It occurs in the form of large granules of a characteristic shape.

### Chlorophyceae

The green algae are characterized by bright green chromatophores, or "chloroplastids," developed in response to the bright light of the sun which in all probability reached the earth in its full intensity for the first time in the Chlorophycean period.

Pigmentation.—As in the higher plants, the chloroplastids of the green algae contain but the four pigments, chlorophyll *a*, chlorophyll *b*, carotin, and xanthophyll, usually designated the "chlorophyll-carotinoid" combination.

Willstätter and Stoll (**47**) investigated the green alga, *Ulva lactuca*, for comparison with the brown algae. They found the

chlorophyll relatively rich in the *b* component. They state: "The ratio of the chlorophyll components in this seaweed deviates, though not far from the average value (about 3), towards a smaller value, just as in the case of the Phaeophyceae." Carotin and xanthophyll are present in relatively greater proportions than in the higher plants, although not to the same extent as in the brown algae.

### Food reserves in orders of the Chlorophyceae.—Siphonales

This group of marine algae evidently is the most nearly related to the next older one, the Chrysophyceae. In this respect it is interesting to note that, in certain members of this order, the stored reserve material consists of globules of oil, suggesting the various subclasses of the yellow-green algae. *Vaucheria*, for instance, produces large amounts of oil in its coenocytic body as well as in the resting zygote. Some investigators think that the oil drops hang on the outside of the chloroplastid, from which they appear to have been exuded. At any rate the oil is usually observed outside the chromatophore. It was made evident by Borodin (7) that the oil drops arise from assimilation. The oil increases in quantity in the light and decreases in the dark. It is thought that the formation of oil is preceded by a carbohydrate. The same thing may be true for the oil of *Bryopsis* and other members of the Siphonales. While *Vaucheria* produces oil (Borodin 7), in another genus (*Dichotomosiphon*) belonging to the same family the stored reserve material consists of starch.

Protein is undoubtedly stored in amorphous form in many cases in the algae, as in the higher plants. Commonly it appears in crystalloid form. "Crystal needles" and sphaerocrystals are ordinary reserve material. These crystalloids are always found in the cytoplasm and only in algae having no pyrenoids in their chromatophores. They have been reported by many workers in the Siphonales and Siphonocladales.

In nearly related genera belonging to the Phyllosiphonaceae both oil and starch appear. In other families, *Udotea*, according to Küster (23), and *Derbesia* and *Dichotomosiphon*, according to Ernst (12), produce more or less abundant starch, which is easily

demonstrable. OLTMANNS (33) would be inclined to call this starch the "earliest visible assimilation product."

### SIPHONOCLADALES

All the marine algae belonging to this order manufacture starch.

### ULVALES

The common green seaweeds, *Ulva*, *Enteromorpha*, and *Monostroma*, and related genera, all produce starch as a food reserve.

### CHLOROCOCCALES

Starch production characterizes these forms, which for the most part live in fresh water. Oil, however, is found in certain unicellular green algae: in *Dunaliella* and *Haematococcus*, members of the Volvocaceae; and in *Chlorococcum* and *Cystococcus*, in the Chlorococcaceae. In the former two examples, SENN (39) believes that the oil may be considered reserve material; in the latter two genera, GERNECK (15) reports oil and starch occurring simultaneously. Here one may assume that the oil is formed from carbohydrates.

### CONJUGALES

In the case of *Spirogyra* and of *Mesotaenium*, and other Desmidiaceae, the resting zygote is filled with a fatty oil. Many investigators have stated that starch, first present in the zygote, disappears and is replaced by oil. Later, at the time of germination, starch reappears. From this it may be concluded that different organs of the same species are capable of storing different materials.

### ULOTRICHALES, CHAETOPHORALES, AND CHARALES

The oil possessed by algae belonging to the Trentepohlieae is believed by SENN (39) to be a reserve material. As a rule all the other members of these three orders produce starch by means of the chloroplastid.

Volutin is produced more or less abundantly in the Volvocaceae, *Tetraspora*, Desmidiaceae, Zygnemaceae, and *Coleochaete*. It may be regarded with certainty as a reserve material.

Mannite has not been observed in green algae.

In a considerable number of the Chlorophyceae the physiological

agreement with higher plants has been ascertained without difficulty, as indicated by the investigations of FAMINTZIN (13) and KRAUS (22). It was found that *Spirogyra* loses its starch in darkness, but that, immediately upon being placed again in the light, it begins to build new starch.

Just as there are starchy seeds and oily seeds in the flowering plants, so there are the same two types of reserve substance in the resting zygote of the green algae. From the green algae up through the flowering plants, therefore, true starch and oil remain as characteristic reserve foods. Pentosan also occurs, but as a secondary reserve of far less importance.

In briefly reviewing the history of pigmentation in the algae, it will be noted that from the beginning there have been present a "chlorophyll-pair," possibly representing the structure elaborated as the food-making constituent of the pigment group, and a "carotinoid-pair," possibly having some auxiliary function, as that of removing the oxygen from chlorophyll *b*, as suggested by WILLSTÄTTER and STOLL. It may be that the pigments phycocyanin, phycoerythrin, and fucoxanthin, each in its turn, had the function of absorbing available rays of light and passing them on to the food-manufacturing group. At the present time almost nothing is known about the pigments and their functions in the algal groups, especially in the Cyanophyceae, Rhodophyceae, and Chrysophyceae. It must be noted, however, that the so-called "chlorophyll-carotinoid" combination of the higher plants had its origin in the algae of the Cyanophycean period, became partly or wholly modified in the Rhodophyceae, and at least appeared in the Phaeophyceae, as the complex structure that has continued apparently practically unchanged throughout all the succeeding geological ages.

A list of reserve materials reported by various authorities is appended:

| | |
|---|---|
| Cyanophyceae: | Glycogen |
| Rhodophyceae: | Floridean starch (KYLIN) |
| Phaeophyceae: | Fucosan (KYLIN) |
| Chrysophyceae: | Oil (KLEBS, BOHLIN, BEIJERINCK) |
| | Leucosin (CZAPEK, PASCHER, WEST and FRITSCH) |
| | Paramylon (GOTTLIEB) |

Chlorophyceae: Oil: in some of the marine forms and in resting zygotes (BORODIN)

Starch: for the first time in the history of plant life, true starch was formed as the product of metabolic activity

**Summary**

Inasmuch as the newer technique in spectral analysis and microchemistry has not yet been extensively applied to studies of pigments and food reserves in the various algal groups, one hesitates to make categorical statements regarding these matters at the present time. While a great amount of work has been done in the past, it has been carried on for the most part by individuals interested in but a small group of algae, or in but a limited phase of the subject. There is need for comparative chemical and physiological work embracing the pigments and products of assimilation in all of the classes of algae, as well as in the higher plants.

In the absence of precise knowledge it may be stated that, in the passing from one algal group to the next (plate I), with every change in the pigment content of the chromatophore there apparently has been a corresponding change in the "metabolic level" of the whole organism. Possibly this change in the chromatophore—morphological perhaps, physiological certainly—has directly or indirectly influenced the type of reserve food products that the organism is able to synthesize.

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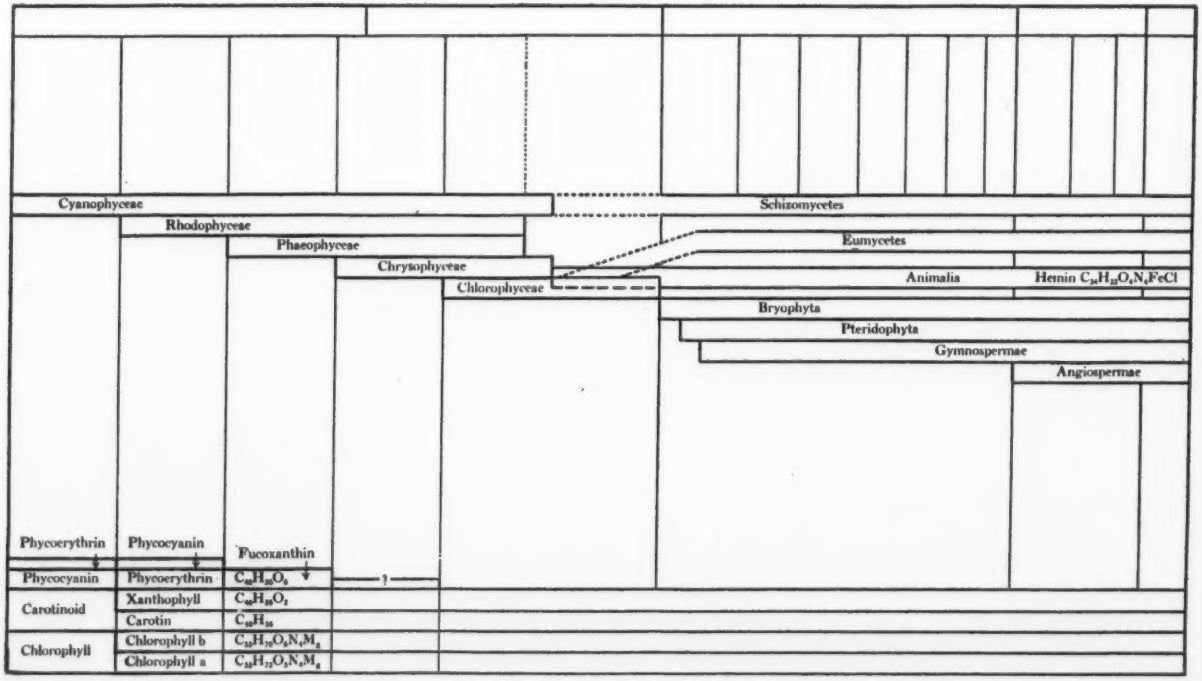
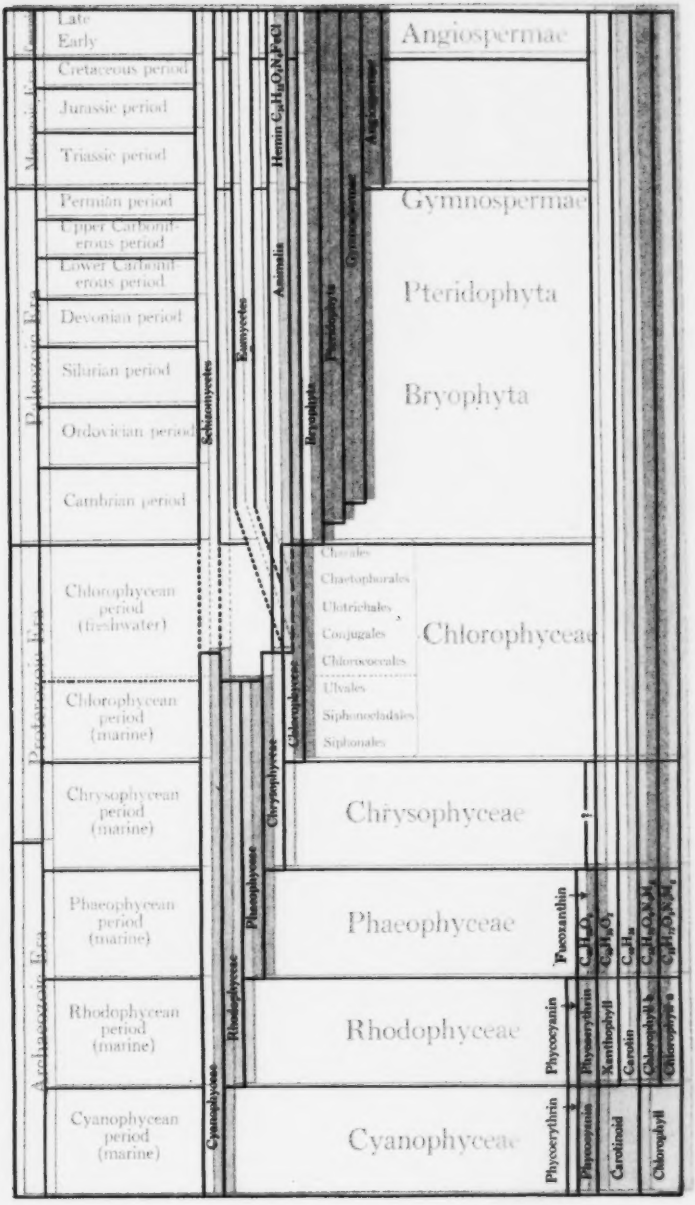
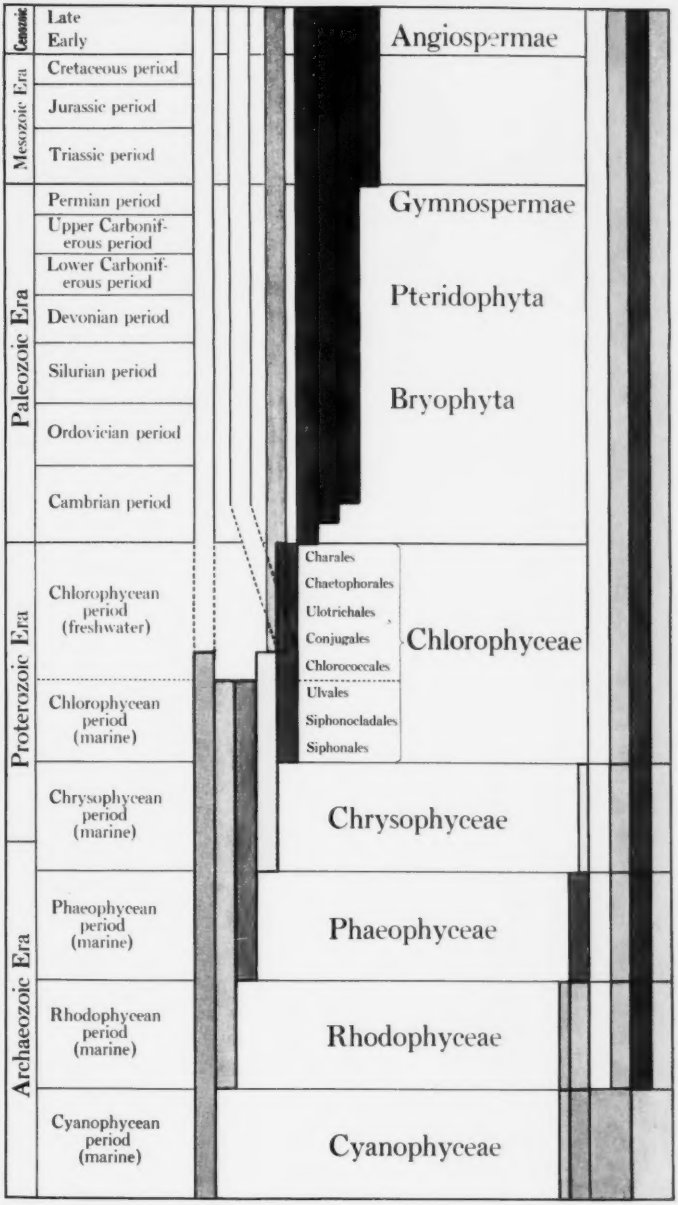

Cyanophyceae
Schizomycetes
Rhodophyceae
Phaeophyceae
Eumycetes
Chrysophyceae
Animalia
Hemin $C_{34}H_{32}O_4N_4FeCl$
Chlorophyceae
Bryophyta
Pteridophyta
Gymnospermae
Angiospermae
Phycoerythrin
Phycocyanin
Fucoxanthin
Phycocyanin
Phycoerythrin
$C_{40}H_{56}O_6$
?
Carotinoid
Xanthophyll
$C_{40}H_{56}O_2$
Carotin
$C_{40}H_{56}$
Chlorophyll
Chlorophyll b
$C_{55}H_{70}O_6N_4Mg$
Chlorophyll a
$C_{55}H_{72}O_5N_4Mg$




A GEOLOGICAL TIME TABLE INCLUDING THE FIVE ALGAL PERIODS

This records data giving in a general way the probable sequence of algae and other plant organisms that lived in past geological ages. Fossil blue-green and red algae are reported from the Archeozoic and Proterozoic eras.

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# NEW OR OTHERWISE NOTEWORTHY COMPOSITAE. IX

EARL EDWARD SHERFF

**Raillardia demissifolia** sp. nov.—Fruticosa forsitan arborescens, ramis pubescentibus conferte usque ad inflorescentiam foliosis. Folia opposita, sessilia, sicca plana vel interdum convoluta, 3-nervia nervis supra impressis, valde patentia vel mox demissa, lanceolato-oblonga, coriacea, faciebus glabrata vel infra sparsissime adpresso-hispida, supra subnitida, marginibus integra vel scabrido-ciliata, apice subobtusa, circ. 1.8–2.7 cm. longa et 5–9 mm. lata. Capitula laxe in panicula ∓1 dm. longa disposita, subnumerosa, pedicellata pedicellis gracilibus glanduloso-hispidis ultimis 0.5–2 cm. longis, ∓8-flora. Involucrum non visum. Achaenia lineari-clavata, atra, adpresse erecto-hispida, corpore 4–5.3 mm. longa.

**Specimens examined:** *Joseph F. Rock*, alt. 6000 ft., Haleakala, Isl. Maui, Hawaiian Isls., October, 1910 (type in Herb. Gray).

**Raillardia reticulata** sp. nov.—Fruticosa vel arborescens, demum 6–7.5 m. alta, valde ramosa ramis purpurascentibus pubescentibusque. Folia opposita, patentia, sessilia, plana vel marginaliter raro subrevoluta, oblonge lanceolata vel lineari-spathulata, basi vix subamplexicaulia apice acuta, coriacea, supra plus minusve subnitida, utrinque adpresse albido-hispida, marginibus ciliata et minutissime paucidenticulata, longitudinaliter circ. 5–7-nervia sed inter nervos plerumque reticulata, 3.5–5.5 cm. longa et nunc subangusta 5–7 nunc latiora 7–13 mm. lata. Capitula paniculata vel rarius paniculato-subcorymbose disposita, saepe numerosissima, conferta, ∓6-flora, pedicellata pedicellis tenuibus glanduloso-hispidis saepius 2–4 mm. longis. Involucrum anguste cylindraceum valde glandulosum sparsim villosum circ. 6–7 mm. longum, bracteis 4–8 non demum separatis. Achaenia nigra, tenuiter lineari-obconica, suberecte albido-hispida vel infra glabrata, circ. 4.5 mm. longa.

**Specimens examined:** *Joseph F. Rock* 8573, shrub, Puunianiau Crater, Haleakala, Isl. Maui, Hawaiian Isls., October, 1910 (2 type sheets in Herb. Gray); *idem* 8590, a tree 20–25 ft. high, alt. 6000 ft.,

above Ukulele, Haleakala, Isl. Maui, Oct. 11, 1910 (Herb. Gray, 3 sheets).

RAILLARDIA MONTANA **longifolia** var. nov.—Rami brunneo-subpurpurascentes. Folia opposita, patentia, subrhomboide ellipticolanceolata, aegre coriacea, non nitida, glabrata, 5- vel 7-nervia, 5–6.5 cm. longa. Capitula circ. 8–10-flora. Achaenia corpore 4.5–5.5 mm. longa.

**Specimens examined:** *Joseph F. Rock* 8603, Kaupo Gap, Haleakala, Isl. Maui, Hawaiian Isls., Oct. 22, 1910 (type in Herb. Gray).

RAILLARDIA PLATYPHYLLA **leptophylla** var. nov.—Folia confertissima, anguste lanceolata, plerumque 6–7 cm. longa et 1–1.5 cm. lata.

**Specimens examined:** *Joseph F. Rock* 8578, on Puunianiau Crater, Haleakala, Isl. Maui, Hawaiian Isls. (type in Herb. Gray).

RAILLARDIA MENZIESII **angustifolia** var. nov.—Folia opposita, linearia vel anguste oblongo-elliptica, 3–5.5 cm. longa et 5–12 mm. lata.

**Specimens examined:** *Joseph F. Rock* 8546, alt. 6000 ft., Haleakala, Isl. Maui, Hawaiian Isls., September, 1910 (type in Herb. Gray); *idem* 8579, summit of Haleakala, October, 1910 (Herb. Gray).

**Raillardia rocki** sp. nov.—Fruticosa, ramis pilosis, purpurascentibus, moderate foliosis. Folia alterna, plana vel subplana, spathulato-oblonga vel superiora oblongo-lanceolata, coriacea et supra nitida, inferne ad basim sessilem saepius sensim angustata, apice subobtusa, marginibus hispidulo-ciliata et ∓3-denticulata, faciebus glabra vel obsolete adpresso-hispida, manifeste 3-nervia nervis supra impressis, 3.5–4.5 cm. longa et 7–9.5 mm. lata. Capitula (∓45 pro unica inflorescentia) paniculate vel subracemose disposita inflorescentia ∓1.9 dm. longa ramis gracilibus villosis bracteatis (bracteis magnis et foliis caulis similibus), ∓14-flora, corollis sulphureo-flavis pappo paululum brevioribus. Involucrum subanguste campanulatum, viride vel demum purpurascens, hispidulum, bracteis 7–9, connatis vel demum interdum pro parte parva separatis. Achaenia nigra, lineari-clavata, basi manifeste angustata, hispida vel inferne glabrata, 4–5 mm. longa.

**Specimens examined:** *Joseph F. Rock* 8601, Laie, Kaupo Gap, Haleakala, Isl. Maui, Hawaiian Isls., Oct. 22, 1910 (type in Herb. Gray).

**Raillardia coriacea** sp. nov.—Fruticosa, caule forsitan decumbens saltem ramis confertissime foliosis ∓4 dm. altis erecta. Folia sessilia, plus minusve terno-verticillata sed superiora alterna, oblongo-linearia, valde reflexa vel superiora adscendentia, coriacea, faciebus glaberrima, supra nitidissima, 3-nervia nervis supra impressis, marginibus revolutis sparsim spinuloso-ciliata et apicem versus 1–4-denticulata, subplana vel longitudinaliter convoluta, 4–5.5 cm. longa et pansa circ. 5–6.5 mm. lata. Capitula numerosa (∓60–80 pro unica inflorescentia), in panicula ∓2 dm. longa disposita, ∓10-flora, pedicellis ultimis tenuibus saepius 3–7 mm. longis, corollis flavis pappo vix aequantibus. Involucrum cylindraceo-campanulatum, hispidulum, purpurascens, 5–6.5 mm. longum, bracteis 6–9 demum plus minusve separatis. Achaenia nigra, oblanceolata, glabra, tantum circ. 3.8–4.2 mm. longa.

**Specimens examined:** *Joseph F. Rock* 8638, Koolau Gap, Haleakala, Isl. Maui, October, 1910 (type in Herb. Gray).

DUBAUTIA LAXA **obovata** var. nov.—Folia obovata, adpresso-hispida, circ. 6–7 cm. longa.

**Specimens examined:** *A. A. Heller* 2902 *pro parte*, Isl. Oahu, Hawaiian Isls. (type in Herb. Kew).

More than twenty years ago, in the course of a monographic study of the genus *Bidens*, my attention was called to the genus *Lipochaeta*, all the recognized species of which are native to the islands of the Pacific Ocean, and certain species of which (for example, *L. micrantha* A. Gray and *L. tenuifolia* A. Gray) through their foliage offer a deceptive resemblance to some species of *Bidens*. Subsequently, Mr. C. N. FORBES, of the Bishop Museum in Honolulu, secured for me a large assemblage of Hawaiian specimens of *Lipochaeta* and most of these were deposited in the Herbarium of Field Museum of Natural History, Chicago, for future study. Since then certain matters concerning *Lipochaeta* have received treatment in my writings. In the main, however, work on the genus was deferred until the summer of 1932. At that time I was urged by Professor OTTO DEGENER to make at once a complete monographic study of the genus, to the end that he might utilize the treatment for his large and monumental Flora Hawaiiensis, now appearing in frequent in-

stallments. A revisional study was at once begun. Professor DEGENER very generously placed at my disposal his many field notes, unpublished portions of manuscript, and a vast number of *exsiccatae*. These last have been determined and labeled, and, whenever the quantities permitted, distributed through Field Museum to the principal herbaria of the world. In all cases where DEGENER has anticipated my conclusions or has been the discoverer of novelties, he has kindly permitted the use of his name in joint authorship. This procedure has been followed, therefore, except in the case of *L. degeneri*, which has been named in his honor (despite his modest protest). Several of the forms treated have been delineated in excellent full-page plates by some of his associated artists for the Flora Hawaiiensis, and various others will likewise be illustrated in the near future.

Most of the world's principal herbaria have lent their entire *Lipochaeta* collections for this research. As a result, every type specimen of real consequence has been made accessible, also many cotypes (the word cotypes being used consistently throughout my writings to connote duplicates of the type, *ex numero et caeteris*). A key to all the species, a list of species excluded, etc. must be deferred, for lack of space, to a subsequent paper in which an extended monographic treatment of the genus will be presented.

**Lipochaeta alata** sp. nov.; *L. calycosa* var. *β*. Hillebr. Fl. Haw. Isls. 207. 1888.—Fruticosa, erecta, circ. 6 dm. alta, ramosa, ramis plus minusve angulatis primum dense scabrido-hispidis demum subglabratis. Folia opposita infra in petiolos breves (4–15 mm. longos) plus minusve alato-marginatos basi saepe aegre connatos contracta, petiolo adjecto 5–9 cm. longa et 3.5–5.5 cm. lata, lamina indivisa deltoideo-ovata vel rhombeo-ovata membranacea (demum saepe perspicue venosa) supra viridia scabridaque subtus pallidiora canescenti-hispidaque, apice acuta vel breviter acuminata, margine acriter duplicato-serrata. Capitula cymose disposita, tenuiter pedicellata pedicellis nunc hispidis nunc glabratis saepius 5–10 mm. longis, radiata, pansa ad anthesin 1.5–2 cm. lata et circ. 7–8 mm. alta. Involucri hispidi bracteae exteriores 4 vel 5 oblongae vel anguste ovatae 2–3.2 mm. longae, interiores latiores saepe paulo longiores, apice angustatae. Flores ligulati plerumque 7–10 interdum 10–14, flavidi, ligula lineares vel elliptico-oblanceolati, apice 2– (vel inter-

dum sub–3–) lobulati, circ. 7–9 mm. longi. Achaenia oblanceolata vel obovata, plus minusve triquetra, faciebus inferne glabrata superne verrucosa hispidulaque, nigra, angulis phellodonta, corpore 2.5–3.2 mm. longa, apice plerumque 2– (saepe imperfecte 3–) aristata aristis 0.2–0.5 mm. longis, inter aristas squamellis erectis coronata.

**Specimens examined:** *Abbé Urbain Faurie* 1007, Waimea, Isl. Kauai, March, 1910 (Herb. Deless., 2 sheets); *A. A. Heller* 2563, along the Hanapepe River, near the Falls, Isl. Kauai, Hawaiian Isls., Jul. 12, 1895 (type, Herb. Field Mus.: cotypes, Herb. Bish. Mus.; Herb. Gray; Herb. N.Y. Bot. Gard.; Herb. Par.; Herb. U.S. Nat.); *idem* 2563*a*, in a thicket, Hanapepe Valley, some three miles above station for no. 2563, *eodem tempore* (Herb. Deless.; Herb. Field Mus.); *H. Mann* and *W. T. Brigham* 542, alt. 2000–3000 feet, Waimea, Isl. Kauai (Herb. Field Mus.; Herb. Gray).

LIPOCHAETA ALATA **acrior** var. nov.—A specie foliis acerrime laciniato-dentatis apice perspicue acuminatis differt.

**Specimens examined:** *U.S. Exploring Expedition under Capt. Wilkes*, Isl. Kauai, Hawaiian Isls., 1840 (type, Herb. U.S. Nat.: cotype, Herb. Gray).

LIPOCHAETA sect. **Macraea** (Hook. *f.*) comb. nov.; *Macraea pro genere* Hook. *f.*, Proc. Linn. Soc. **1**:278. 1845; *etiam* Trans. Linn. Soc. **20**:209. 1847; *Trigonopterum* Steetz *in* Anders. Enum. Pl. Galap., Trans. Acad. Sc. Stockh. **1853**:161. 1853.—BENTHAM & HOOKER (Gen. Plant. **2**:373. 1873) recognized two sections in the genus *Lipochaeta*, namely, *Microchaeta*[1] (*pappi aristis prominulis*) and *Aphanopappus* (*pappo ad coronulam minutam reducto vel omnino deficiente*). To the latter they referred *Macraea* Hook. *f.*, a genus founded upon a highly anomalous Galapagos Island species, *L. laricifolia* (Hook. *f.*) A. Gray.[2] A study of all the known species of *Lipochaeta*, however, shows that their differentiation in nature has evolved more in respect to foliage and to grosser characters of capitula than in respect to achenes. Proper recognition of this fact compels placing the anomalous *L. laricifolia* apart from each of the

[1] Spelled by them *Microchaete*, but clearly a mere reduction from generic rank of NUTTALL'S *Microchaeta* (Trans. Amer. Philos. Soc. ser. II, **7**:450. 1841).

[2] All other species of *Lipochaeta* as recognized since the revision by ASA GRAY (Proc. Amer. Acad. **5**:129–131. 1861) are native to the Hawaiian Islands except *L. lifuana* Hochr., of the Loyalty and New Hebrides Islands.

two sections maintained by BENTHAM & HOOKER and according to it a separate sectional classification.

**Lipochaeta acris** sp. nov.—Fruticosa, erecta, ramosa, scabrida setis brevibus adpressis. Folia opposita breviter petiolata petiolis vix marginatis 0.5–1 cm. longis, petiolo adjecto 8–12 cm. longa et 3–7 cm. lata, simplicia, ovata vel anguste rhombeo-ovata, membranacea, basi late vel sublate cuneata, apice moderate vel longe acuminata, marginibus acriter perspicueque duplicato- vel laciniato-dentata dentibus 4–7 mm. longis apice subulatis. Capitula cymose disposita, tenuiter pedicellata pedicellis saepius 1–3 cm. longis, radiata, pansa ad anthesin 2.2–2.5 cm. lata et circ. 7–8 mm. alta. Involucri hispiduli bracteae exteriores circ. 5, oblongo-lineares vel ovatae, superne saepe angustatae vel acuminatae 3–5 (rarius –7) mm. longae, quam interiores plerumque paulo longiores. Flores ligulati 10–14, flavidi, ligula oblongi vel elliptico-oblanceolati, apice minute circ. 3-denticulati, 7–12 mm. longi. Achaenia cuneato-obovata, atra, plus minusve triquetra, faciebus glabra nitidaque, duobus marginibus plus minusve spinulosa, corpore circ. 2.5 mm. longa et circ. 1.5 mm. lata, apice truncato breviter biaristata et minute setulosa.

**Specimens examined:** *H. Mann* and *W. T. Brigham* 540, alt. 2000–3000 feet, Waimea, Isl. Kauai (type, Herb. Field Mus.: cotypes, Herb. Deless., 2 sheets; Herb. Gray; Herb. Mo. Bot. Gard.).

**Lipochaeta forbesii** sp. nov.—Fruticosa, erecta, omnino brevissime sed dense adpresso-hispidula, ramosa ramis subteretibus griseo-brunneis. Folia petiolata petiolis laciniate plus minusve alato-marginatis circ. 1.5–2 cm. longis, petiolo adjecto 5–9 cm. longa, plerumque incise 3–5-lobata, membranacea, lobis acriter vel etiam laciniate dentatis apice acutis vel acuminatis terminali multo majore rhombeo-ovato ad oblongo-lanceolato. Capitula numerosa cymose disposita, pedicellata pedicellis plerumque 0.5–2.5 cm. longis, radiata, pansa ad anthesin ∓ 2 cm. lata et circ. 5–6 mm. alta. Involucri hispidi bracteae exteriores 4–6, oblongo-lanceolatae vel linearioblongae, apice plus minusve acutae, 2–3 (raro –3.5) mm. longae. Achaenia polymorpha, atra, exteriora moderate vel late cuneata, 3–4-gona, angulis interrupte alata alarum segmentis saepe erecte interneque hamosis vel rostratis, faciebus praecipue superne spinu-

loso-verrucosa, corpore 2.5–3 mm. longa, apice breviter pauciterque squamelliformi-aristata.

**Specimens examined:** *C. N. Forbes* 1916*M*, Nuu, south slope of Haleakala, Isl. Maui, Hawaiian Isls., Mar. 9, 1920 (type, Herb. Field Mus.).

Named for the late Mr. FORBES who, during the last few years preceding his death, sent many specimens of *Lipochaeta* and *Bidens* to the Herbarium of Field Museum of Natural History as material for this research. As to foliage, the habit suggests that of *L. rockii* var. *subovata* (*vide* p. 101), of the Island of Molokai, but in the abundance and size of capitula the resemblance to *L. kahoolawensis* (*vide* p. 98), of the Island of Kahoolawe, is closer.

**Lipochaeta degeneri** sp. nov.—Fruticosa, plus minusve erecta, multum ramosa ramis virgatis griseis vel rubido-griseis teretibus subcanescentibus (setis albidis valde adpressis), saltem 3–4 dm. alta. Folia vix petiolata petiolis ∓1.5 mm. longis, spathulata vel lineari-oblanceolata, apice obtusa vel rotundata, membranacea, viridia, subrevoluta, integra vel obscure denticulata, 0.7–3 cm. longa et 2.5–5.5 mm. lata. Capitula numerosa tenuiter pedunculata pedunculis solitariis vel 3–5-adgregatis 1–3 cm. longis, radiata, pansa ad anthesin ∓ 1 cm. lata et circ. 5 mm. alta. Involucri hispidi bracteae subaequales, exteriores circ. 4, ovatae, obtusae, circ. 2 mm. longae. Flores ligulati 4–6, subflavidi, ligula oblongi, apice plerumque tridenticulati, circ. 5–6 mm. longi. Achaenia atra, exteriora cuneata, triquetra, angulis suberoso-alata, faciebus muriculata, corpore 1.5–2 mm. alta, apice erecte 2-3–aristulata et plus minusve setosa; interiora obcompressa, anguste cuneata, exalata, faciebus inferne glabra superne erecto-setulosa, corpore circ. 2 mm. longa, apice truncato erecte fimbriata et plerumque biaristulata.

**Specimens examined:** *Otto Degener* 4198, hot, arid, boulder-covered plain near sea, near southwest point of Isl. Molokai, May 16, 1928 (type, Herb. Field Mus.); *C. N. Forbes* 59*Mo*, Kalaeokalaau, Isl. Molokai, June, 1912 (Herb. Field Mus.); *J. F. Rock* 10288, *eodem loco*, 1910 (Herb. Gray).

LIPOCHAETA INTEGRIFOLIA **argentea** var. nov.—A specie foliis minoribus (plerumque 6–14 mm. longis et 1–4 mm. latis) saepius oblongo-linearibus perspicue canescentibus vel argenteis, ramulis plerumque canescentibus differt.

**Specimens examined:** *Abbé U. Faurie* 944, Wailuku, Isl. Maui, August, 1909 (Herb. Brit. Mus.); *Wm. Hillebrand*, above Maalae Bay, Isl. Maui (Herb. Berl.); *A. S. Hitchcock* 15131, sandy beach, western part of Isl. Molokai, Oct. 12, 1916 (U.S.); *Mann* and *Brigham* 371, on sandy isthmus, Isl. Maui (type, Herb. Field Mus.: cotypes, Herb. Deless.; Herb. Field Mus.; Herb. Gray; Herb. U.S. Nat.); *U.S. Exploring Exped. under Capt. Wilkes*, sand hills, Isl. Maui, 1840 (Herb. N.Y. Bot. Gard.; Herb. U.S. Nat.).

*Lipochaeta integrifolia* (Nutt.) A. Gray, of which I have had opportunity to examine numerous specimens, is distributed over the Islands of Molokai, Oahu, Kauai, Laysan, and Kure of the Hawaiian Archipelago (including the Leeward Islands).[3] The var. *argentea*, which for many years has been confused with the species proper, is known to me only from the Islands of Maui and Molokai.

LIPOCHAETA INTEGRIFOLIA **gracilis** var. nov.—Habitu gracilis, ramis (siccis) brunneo-atris elongatis tenuissimis, foliis moderate canescentibus linearibus tantum 7–12 (raro –16) mm. longis et 1–2 mm. latis, basi saepe in petiolum parvum angustata, nonnullis principalibus sub medium utrinque 1-dentatis vel parce 1-lobulatis.

**Specimens examined:** *Charles Gaudichaud* (*Freycinet's Voyage*) 217, Hawaiian Isls., September-October, 1836 (type, Herb. Gray: cotype, Herb. Par.); *idem, sine num., eodem loco* (Herb. Berl.; Herb. Par.).

Easily recognized by its slender habit, narrowly linear leaves, and by the fact that these are often provided with a conspicuous tooth or even small lobe on each lateral margin.

LIPOCHAETA INTEGRIFOLIA **major** var. nov.—Dense adpresso-hispidula sed non perspicue argentea, ramis (ramulis) saepe 1.2 dm. longis, foliis usque ad 4 cm. longis et 1 cm. latis, spathulatis, saepe crenatulatis, apice saepius obtusissimis, basi saepe breviter petiolatis petiolis usque ad circ. 5 mm. longis.

**Specimens examined:** *Guppy*, rocky sea-coast, Isl. Oahu, 1897 (Herb. Kew); *A. A. Heller* 2092, on the old lava flow back of Diamond Head, Isl. Oahu, Apr. 8, 1895 (type, Herb. Gray: cotypes, Herb. Deless.; Herb. Field Mus.; Herb. Mo. Bot. Gard.; Herb. N.Y. Bot. Gard.; Herb. Par.; Herb. U.S. Nat.).

[3] Laysan and Kure are here cited on the authority of CHRISTOPHERSEN & CAUM, Vascular Pl. Leeward Isls., Bishop Mus. Bull. 81. 39. 1931.

A much coarser plant than the species proper. Not to be confused with the unnamed var. $\beta$ of HILLEBRAND's Flora of the Hawaiian Islands (p. 208. 1888), the type of which was collected at Kailua of the same Island and is still extant (Herb. Berl.). This latter is found to be merely a scarcely distinguishable form of the species itself.[4]

LIPOCHAETA INTEGRIFOLIA **megacephala** Degener & Sherff var. nov.—Habitu var. *majori* similis sed differt foliis interdum longe petiolatis (usque ad 1.5 cm.), capitulis pansa ad anthesin 1.5–2 cm. latis.

**Specimens examined:** *E. Christophersen* 1400, in sand at alt. ∓5 m., Kaena Point, Isl. Oahu, Dec. 14, 1930 (type, Herb. Field Mus.); *Otto Degener, K. K. Park,* and *W. Hirai* (*Degener* distrib. no.) 4179, on arid clay soil near the ocean, Kaena Point, Isl. Oahu, Mar. 21, 1931 (Herb. Field Mus.); *Guppy,* Kaena Point, Isl. Oahu, 1897 (Herb. Kew).

This and the foregoing varieties may be separated from the species itself according to the following key:

a. Folia 1–2 mm. lata, nonnulla principalia sub medium utrinque 1-dentata vel parce 1-lobulata. . . . . var. *gracilis*
a. Folia plerumque latiora (nisi pro var. *argentea*), principalia integra vel obscure denticulata
  b. Folia usque ad 4 cm. longa et 1 cm. lata
    c. Capitula pansa ad anthesin circ. 1.2 cm. lata. . . . var. *major*
    c. Capitula pansa ad anthesin 1.5–2 cm. lata. . . var. *megacephala*
  b. Folia plerumque 0.6–2.5 cm. longa et 1–7 (raro –9) mm. lata
    c. Folia plerumque 1–2.5 cm. longa et 3–7 (raro –9) mm. lata *L. integrifolia* sensu stricto
    c. Folia plerumque 6–14 mm. longa et 1–4 mm. lata var. *argentea*

LIPOCHAETA SUCCULENTA var. **decurrens** (A. Gray) comb. nov.; *L. lanceolata* Nutt., Trans. Amer. Phil. Soc., ser. II, 7:451. 1841; *L. australis* var. *decurrens* A. Gray, Proc. Amer. Acad. 5:129. 1861; *L. connata* var. *decurrens* (A. Gr.) Hillebr. Fl. Haw. Isls. 206. 1888; *L. connata* var. *littoralis* Hillebr. *loc. cit.; L. variolosa* Lévl., Fedde Repert. 10:122. 1911.

[4] As is also an additional specimen by *C. N. Forbes,* no. 615*Mo,* from Papoia, a small island near Kailua, April, 1914 (Herb. Field Mus.).

NUTTALL (*loc. cit.*) appears to have been the only observer to note the peculiar herbaceous habit of this plant: "Branches long and trailing, prostrate and ascending." The color, scabridity, and size of the leaves vary remarkably and, if it were not for the large assortment of interconnecting herbarium specimens available at present for study, one might well be misled into assuming that several distinct varieties were represented.

LIPOCHAETA SUCCULENTA **trifida** var. nov.—Varietati *decurrenti* similis sed foliis acriter trifidis vel trilobatis differt.

**Specimens examined:** *C. N. Forbes* 397*Mo*, Manawai, Isl. Molokai, Hawaiian Isls., August, 1912 (3 type sheets, Herb. Field Mus.).

LIPOCHAETA SUCCULENTA **barclayi** var. nov.—Fruticosa, saepe robusta, 9–12 dm. alta, caulibus siccis 4–6 mm. crassis. Folia nunc elliptico-oblonga nunc rhombeo-ovata, usque ad 1 dm. longa et 4.3 cm. lata, ad apicem mucronulatum plerumque rotundo-obtusa, margine leviter subremoteque denticulata dentulis subulatis vel mucronulatis, sicca subnitida et viridi-brunnea.

**Specimens examined:** *George Barclay* 1327, shrub 3 or 4 ft. high, in loam soil, hills, Isl. Atooi (Isl. Kauai), Hawaiian Isls., July, 1837 (type, Herb. Brit. Mus.); *C. N. Forbes* 71*K*, Kololau Trail, Isl. Kauai, Jul. 19, 1909 (Herb. Field Mus., 3 sheets).

LIPOCHAETA SUCCULENTA **angustata** var. nov.—Folia oblonge linearia vel lineari-lanceolata, principalia nunc 6–8 cm. longa et 3–5 mm. lata nunc paulo longiora et 8–12 mm. lata.

**Specimens examined:** *C. N. Forbes*, Isl. Kauai, 1909 (2 type sheets, Herb. Field Mus.).

The four varieties here given for *L. succulenta* (H. & A.) DC. may be separated from the species proper[5] according to the following key:

[5] The type of the species was collected, according to HOOKER & ARNOTT (Bot. Beechey's Voy. 87. 1832) on the Island of Oneeheow, now commonly known as Niihau. The type sheet (Herb. Kew) gives Oahu, however. Since the species is not otherwise known to me from Oahu, it may be that the plant actually did come from Niihau. Or possibly specimens were collected on both islands, the one from Oahu at first escaping the notice of investigators, while the one from Niihau failed to be preserved to the present day. Very probably HOOKER himself later saw the plant ascribed to Oahu, for its sheet bears the stamp of his private herbarium.

a. Folia oblonge linearia vel lineari-lanceolata.....var. *angustata*
a. Folia latiora
b. Folia plerumque trifida vel trilobata.....var. *trifida*
b. Folia indivisa
c. Folia apice subacuta ad acuminata; plantis molokaiensibus var. *decurrens*
c. Folia apice plerumque obtusa vel rotundata; plantis kauaiensibus niihauensibusque
d. Folia rhomboide vel oblonge obovata vel oblanceolata *L. succulenta* sensu stricto
d. Folia nunc elliptico-oblonga nunc rhombeo-ovata var. *barclayi*

LIPOCHAETA LAVARUM **ovata** var. nov.—Folia principalia ovata vel interdum subrhombeo-ovata, crenato-serrata, canescentia, apice obtusa, triplinervia nervis divergentibus, subtus venosissima, basi in petiolum saepius 1–1.5 cm. longum angustata, petiolo adjecto 4.5–6 cm. longa et 1.8–2.5 cm. lata.

**Specimens examined:** *C. N. Forbes* 2015*M*, Puu Onole, south side of Haleakala, Isl. Maui, Mar. 18, 1920 (Herb. Field Mus.); *J. F. Rock* 8674, below the crater at Kahikinui, Isl. Maui, November, 1910 (type, Herb. Gray: cotype, Herb. Field Mus.).

*Forbes* 2020*M*, from much the same vicinity in southeastern Maui (Kamana, southern slope of Haleakala), has foliage not really separable from that of *L. lavarum* proper. Thus *L. lavarum* is seen to be present in the vicinity whence came my type. Indeed the cited cotype sheet has, besides two sprays of the ovate-leaved form, one spray with numerous smaller and narrower leaves hardly atypic for *L. lavarum*. The ovate-leaved form, however, is known from nowhere else in the Hawaiian Islands and appears best construed as an endemic variety.

LIPOCHAETA LAVARUM **salicifolia** var. nov.; *L. lavarum* var. β, Hillebrand Fl. Haw. Isls. 207. 1888.—Folia secundaria oblongo-linearia, integra, valde membranacea, aegre venosa, supra viridia infra aegre pruinosa, 3.5–7.5 mm. lata.

**Specimens examined:** *E. Bishop*, near Lahaina, Isl. Maui, Hawaiian Isls. (type, Herb. Berl.); *C. N. Forbes* 2270*M*, Lahaina, Isl. Maui, May 8, 1920 (Herb. Field Mus.; *forma vix typica*).

HILLEBRAND'S type is a small flowering branch, less than 1.5 dm. long. This subdivides near the base into branchlets (besides a terminal peduncle), and the leaves present, although numerous, include none of the larger primary or principal leaves which on some specimens of *L. lavarum* occur only on the larger branches. Hence my leaf description must needs be regarded as incomplete. The leaves are thin or membranaceous and appear comparatively so green on the upper surface as to seem glabrous; but under a lens, abundant appressed, white hairs can be seen.

*Forbes 2270M* was collected in the type locality. The specimen cited is a handsome one, some 5 dm. long and with numerous leaves and various flowering and fruiting heads. It is probably to be construed as a slightly atypic form of this variety. The leaves are somewhat more veiny, at the apex more often acute, and the few principal leaves present measure up to 12 mm. in width. The larger the leaves the more tendency there is for them to become crenate-denticulate.

LIPOCHAETA LAVARUM **skottsbergii** var. nov.—Folia numerosa, strigoso-canescentia, linearia, 2–3.5 cm. longa et tantum 1.5–3 mm. lata.

**Specimens examined:** *Frederick Debell Bennett* 43, Isl. Maui, 1833–1836 (type, Herb. Berl.).

Through this variety *L. lavarum* is seen to approach, in foliage, the erect forms of *L. integrifolia*. The name is chosen in honor of C. J. F. SKOTTSBERG, Gothenburg, Sweden, who studied the type in 1925 and treated it as a narrow-leaved form of *L. lavarum*.

LIPOCHAETA LAVARUM **hillebrandiana** var. nov.; *L. lavarum* var. γ, Hillebrand Fl. Haw. Isls. 208. 1888.—Humilis, fruticosa, patens, divaricate ramosa. Folia secundaria lineari-oblonga vel spathulata, membranacea vel vix crassiuscula, sicca coriacea, triplinervia, canescentia, integra, apice obtusa vel quidem rotundato-obtusa, tantum 2–3 cm. longa et 3–7 mm. lata. Achaenia interrupte alata.

**Specimens examined:** *Wm. Hillebrand*, on rocks near sea, Lahaina, Isl. Maui (type, Herb. Berl.); *idem*, gulch back of Lahaina, August, 1870 (Herb. Berl., *cum specie ipsa commixta*); *Mann* and *Brigham* 374, mountain above Maalaea Bay, western Maui (Herb. Field Mus.; Herb. Gray); *Jules Remy* 277, Isl. Hawaii, 1851–1855 (Herb. Gray; Herb. Par.).

The HILLEBRAND herbarium (now incorporated in Herb. Berl.) has five specimens on the type sheet, four of them almost devoid of leaves. An additional sheet, however, bears two sprays of the species proper and one of this variety, all three coming from the type vicinity. HILLEBRAND himself appears to have overlooked this supplementary specimen of his variety. It bears among the numerous secondary leaves several of the larger primary or principal leaves, and these are 10–12 mm. wide but less than 5 cm. long. HILLEBRAND cited Maalaea Bay as a second locality for this variety. *Mann* and *Brigham* 374, from a mountain above Maalaea Bay, apparently has the more shrubby, dwarfed, and divaricately branching habit of the type material, but the leaves average nearly twice as long as in the type, and display in this respect an approach to those of the species proper.

LIPOCHAETA LAVARUM **longifolia** var. nov.—A specie internodiis forsitan elongatioribus foliis principalibus petiolo adjecto 7–13 cm. longis differt.

**Specimens examined:** *G. C. Munro* 202, Maunalei Valley, Isl. Lanai, Hawaiian Isls., Mar. 9, 1915 (type, Herb. Bish. Mus.).

*L. lavarum* (Gaud.)DC. and the foregoing varieties may be distinguished by the following key:

a. Folia principalia linearia, tantum 1.5–3 mm. lata
var. *skottsbergii*

a. Folia principalia latiora
b. Folia principalia plus minusve ovata, 1.8–2.5 cm. lata
var. *ovata*

b. Folia principalia angustiora
c. Folia secundaria numerosa, oblongo-linearia vel spathulata
d. Folia secundaria 2–3 cm. longa.....var. *hillebrandiana*
d. Folia secundaria plerumque 4–7 cm. longa..var. *salicifolia*

c. Folia secundaria plerumque non numerosa, principalibus plus minusve lanceolatis similia
d. Folia principalia petiolo adjecto 7–13 cm. longa
var. *longifolia*

d. Folia principalia petiolo adjecto 2.5–8 cm. longa
*L. lavarum* sensu stricto

LIPOCHAETA SUBCORDATA **populifolia** var. nov.—A specie differt foliis moderate vel late ovato-cordatis vel ovato-subcordatis.

**Specimens examined:** *G. C. Munro* 670, Maunalei Valley, Isl. Lanai, June 18, 1918 (type, Herb. Field Mus.: cotype, Herb. U.S. Nat.).

LIPOCHAETA SUBCORDATA **membranacea** var. nov.—A specie foliis ovato-subcordatis vel subcordato-deltoideis, sparsim adpresso-hispidis itaque perspicue membranaceis utrinque viridibus.

**Specimens examined:** *E. Bishop* 14, back of Lahaina, western Maui (type, Herb. Berl.).

The type was originally in HILLEBRAND'S private herbarium and was the basis of his distributional note, "Maui! back of Lahaina (leaves deltoid, flower-heads larger)."

In *L. subcordata* A. Gray, the leaves tend to be narrowly deltoid, often suggesting in outline a moderately narrow isosceles triangle. The leaves are conspicuously and densely whitish-hispid beneath. The leaves of var. *populifolia* are broader in outline, much like those of *Populus deltoides* Marsh. The leaves of var. *membranacea*, of which I have seen only the small branch on the type sheet, are intermediate in outline but are noticeable for their sparse hispidity, this latter character being associated with more membranaceous texture and a closer similarity in color between the upper and lower surfaces. Much variation also occurs, within the species proper, as to size of leaves, length of internodes, and size of heads, but these variations appear in this species much too inconstant to serve as a basis for the delimiting of varieties.

LIPOCHAETA LOBATA var. **hastulata** (Hook. & Arn.) comb. nov.; *Verbesina hastulata* Hook. & Arn. Bot. Beechey Voy. 87. 1832; *L. hastulata* (Hook. & Arn.) DC. Prodr. 5:611. 1836.—ASA GRAY had labeled the type sheet, "*Lipochaeta australis* A. Gr. var. *lobata*." This is doubtless to be explained by the fact that he had already reduced *L. lobata* (Gaud.)DC. to a varietal rank and was disinclined to reduce the plant under observation to still lower rank, hence the equation of the two. It is doubtful, however, whether out in nature these merge.

The type (Herb. Kew) came from some locality on the Island of Oahu. With it must be associated a specimen collected by *Otto*

*Degener, K. K. Park*, and *W. Hirai*, no. 4181, among rocks, grass, and weeds on talus in dry region, along coast 2 miles east of Kaena Point toward Kawaihapai, Isl. Oahu, Mar. 22, 1931 (Herb. Field Mus.; *forma vix typica*).

LIPOCHAETA LOBATA var. **denticulata** (Wawra) comb. nov.; *L. calycosa* A. Gray, Proc. Amer. Acad. **5**:130. 1861; *L. australis* var. *denticulata* Wawra, Flora **56**:77. 1873.

LIPOCHAETA LOBATA **leptophylla** Degener & Sherff, var. nov.—Folia lanceolata vel lanceolato-linearia, saepe attenuato-acuminata, usque ad 8 (raro 9) cm. longa. Involucri bracteae ovatae, apice plus minusve acuminatae.

**Specimens examined:** *C. N. Forbes* 2024*O*, at Kolekole Pass, Waianae Range, Isl. Oahu, Feb. 1–2, 1915 (3 type sheets, Herb. Field Mus.); *idem et Dean Lake* 2274*O*, talus slopes near Kaena Point, Isl. Oahu, Dec. 16, 1915 (Herb. Field Mus.); *C. N. Forbes* and *J. C. Bridwell* 2476*O*, ridge between Niu and Wailupe, Isl. Oahu, Apr. 11, 1917 (Herb. Bish. Mus.; Herb. Field Mus.); *J. F. Rock* 17122, Wailupe Valley, Isl. Oahu, Apr. 14, 1918 (Bish.).

LIPOCHAETA LOBATA var. **aprevalliana** (Dr.) comb. nov.—*L. aprevalliana* Drake del Cast. Illustr. Fl. Ins. Mar. Pacif. 71. *tab.* 34. 1886.

LIPOCHAETA LOBATA **albescens** var. nov.—Folia saepe 8–13 cm. longa, pallida, coriacea, supra subnitida et subglabrata, petiolis ac nervis principalibus etiam ramulis et pedicellis et involucri bracteis (late ovatis) demum stramineo-albis.

**Specimens examined:** *A. A. Heller* 2021, on steep slopes at Diamond Hill (Diamond Head), Isl. Oahu, Mar. 28, 1895 (type, Herb. Field Mus.: cotypes, Herb. Gray; Herb. N.Y. Bot. Gard.; Herb. U.S. Nat.).

LIPOCHAETA LOBATA **grossedentata** Degener & Sherff, var. nov.—Folia tenera, oblongo-lanceolata, vix petiolata petiolis circ. 2–3 mm. longis, principalia 6–8 cm. longa, marginibus grosse dentata, apice acuta. Capitula pansa ad anthesin circ. 2.5–2.9 cm. lata, ligulis pulchre aureis anguste obovatis circ. 5.5–7 mm. latis apice manifeste bilobulatis.

**Specimens examined:** *Otto Degener, K. K. Park*, and *Wm. Bush*, on rather dry, rocky, partly shaded slope in gulch north of middle

of ridge between Puu Pane and Puu Kamaohanui, Isl. Oahu, Hawaiian Isls., Jan. 10, 1932 (type, Herb. Field Mus., 2 sheets: cotype, Herb. Kew).

LIPOCHAETA LOBATA **hastulatoides** Degener & Sherff, var. nov.—Folia breviter alato-petiolata, ovata vel oblongo-ovata, subcoriacea, acriter serrata, apice saepius subacuta, omnino 3–6.5 cm. longa.

**Specimens examined:** *Otto Degener* 4305, Pohakea Gulch, southeasternmost part of West Maui, Hawaiian Isls., Jul. 11, 1927 (type, Herb. Field Mus., 2 sheets: cotypes, Herb. Berl.; Herb. Brit. Mus.; Herb. Gray; Herb. Kew; Herb. N.Y. Bot. Gard.).

Interesting as being the only form of *L. lobata* known from the Island of Maui. The leaf characters place the variety between var. *hastulata* and var. *denticulata*. The general aspect of the foliage is deceptively like that of some species of *Prunus* and *Pyrus* in the family Rosaceae.

LIPOCHAETA LOBATA **maunaloensis** var. nov.—Folia principalia 4–7 cm. longa, valde membranacea, manifeste hastato-trilobata lobis lateralibus late oblongis ad subrotundis, terminali elongato elliptico-oblongo.

**Specimens examined:** *C. N. Forbes 7Mo*, on Mauna Loa, Isl. Molokai (*nec alibi*), Hawaiian Isls., June, 1912 (type, Herb. Field Mus.).

The varieties of *L. lobata* may be severally distinguished from the species proper according to the following key:

a. Folia (raro 8–10 cm. longa) et ramuli et involucri bracteae virides vel subvirides
  b. Folia basi plus minusve cordata.....var. *aprevalliana*
  b. Folia basi cuneata rotundatave
    c. Folia plus minusve hastulata
      d. Folia principalia saepe 10–12 cm. longa et 4–6 cm. lata; involucri bracteis exterioribus demum 5–7 mm. longis
        var. *hastulata*
      d. Folia principalia plerumque 3–6 interdum usque ad 8 (rarius 10) cm. longa
        e. Folia perspicue lobata, lobo principali anguste ovato vel rhombeo-ovato, planta molokaiensi
          var. *maunaloensis*

e. Folia aegre vel moderate lobata, lobo principali anguste deltoideo-oblongo, planta oahuensis
*L. lobata* sensu stricto

c. Folia non (vel rarissime sub-) hastulata
d. Omnia vel nonnulla folia ovata, involucri bracteis exterioribus oblongo-ovatis vel obovatis apice rotundo-obtusis vel subacutis
e. Petioli alati . . . . . var. *hastulatoides*
e. Petioli exalati . . . . . var. *denticulata*
d. Folia lanceolata vel lanceolato-linearia, serrata vel denticulata, involucri bracteis exterioribus ovatis apice plus minusve acuminatis . . . . . var. *leptophylla*
d. Folia oblongo-lanceolata, grosse dentata
var. *grossedentata*

a. Folia (saepe 8–13 cm. longa) pallida, ramulis involucri bracteisque albescentibus . . . . . var. *albescens*

×**Lipochaeta procumbens** Degener & Sherff, hybr. nov.—Fruticosa, prostrata, ramosa, minute adpresso-hispidula, caulibus elongatis angulatis flexilibus, ∓8 dm. longis. Folia inferne in basim petioloideam cuneato-angustata, principalia omnino 3–7 cm. longa, rhombeo-oblonga, apice obtusa vel subacuta, sparsim subobsoleteque serrulata, crassiuscula, supra manifeste 3-nervia, infra elevato-venosa. Capitula tenuissime pedunculata pedunculis saepe 3–7 cm. longis, laxe disposita, radiata, pansa ad anthesin circ. 1.5 cm. lata et circ. 6 mm. alta. Involucri bracteae exteriores 4 vel 5, late ovatae ad apicem obtusae, 2–3.2 mm. longae (rarius 1 vel 2 minimae oblongae ∓1.5 mm. longae). Flores ligulati aurei 8–10, ligula anguste obovati, apice 2–3-denticulati, ∓7 mm. longi. Achaenia deficientia; ovariis sterilibus.

**Specimens examined:** *Otto Degener, K. K. Park,* and *W. Hirai* (*Degener* distrib. no.) 4178, forming mats on the loose lava rock on arid plain near ocean, Kaena Point, Isl. Oahu, Hawaiian Isls., Mar. 21, 1931 (3 type sheets, Herb. Field Mus.: cotypes, Herb. Kew; Herb. Munich); *Degener* 4303, *eodem loco,* Jan. 31, 1932 (Herb. Field Mus.).

The type specimens were growing among specimens of *L. lobata* var. *denticulata* and *L. integrifolia* var. *megacephala,* and were

suspected by DEGENER of being hybrids between those two varieties. His suspicion appears to be confirmed by the intermediate nature of the main diagnostic characters studied, also by the fact that the several hundred ripe capitula examined have invariably abortive ovaries, not a single achene having been matured.

**Lipochaeta profusa** sp. nov.—Fruticosa, erecta, omnino sed dense hispidula, ramosa ramis subteretibus. Folia opposita inferne abrupte in basim connatam acriter dentatam 8–12 mm. latam contracta, principalia circ. 7–9 cm. longa et 3.5–4.5 cm. lata, margine acriter sed irregulariter serrata, apice breviter acuminata, subtus perspicue elevato-venosa. Capitula cymose disposita, numerosa (unico ramo interdum quidem 50–75 capitula subtendente) pedicellis (ramulis ultimis) plerumque usque ad 2 cm. longis, radiata, pansa ad anthesin ∓15 mm. lata et 5–6 mm. alta. Involucri hispiduli minimi (demum tantum circ. 3 mm. alti et supra tantum circ. 3–3.5 mm. lati) bracteae exteriores 3 vel 4, ovatae, subacutae vel saepius abrupte acutae vel etiam breviter acuminatae, siccae apice plus minusve atrae, 2–3.2 mm. altae. Flores ligulati ∓8, flavi, ligula elliptico-oblanceolati, apice plus minusve bidenticulati, circ. 7–8 mm. longi. Achaenia exteriora brunneo-grisea vel atra, 3–4-gona, angulis interdum interrupte cartilagineo-alata, faciebus levia vel valde verrucosa, cuneata, corpore 2–2.2 mm. alta et supra 1–1.3 mm. lata, apice erecte spinuloso-setosa et erecte pauciaristata aristis brevibus et antrorsum hispidulis.

**Specimens examined:** *Mrs. Isabella Sinclair*, Isl. Hawaii, Hawaiian Isls., *commun.* January, 1885 (type, Herb. Kew).[6]

A species close to *L. alata* (*vide* p. 81), from which it differs in its leaf bases and inflorescence. *L. alata* has the leaves narrowed below into a manifestly winged petiole, while in *L. profusa* the leaf bases (which are distinctly connate), although amounting to a contraction of the blade, are about 8–12 mm. wide and would not be considered as being winged petioles. Furthermore, *L. profusa* has the inflorescence much more decompound and, in the species proper, the heads are comparatively minute.

[6] The type label gives "Hawaii," whence it is assumed that the island by that name, rather than the whole archipelago, later commonly known as Territory of Hawaii, was meant.

The type sheet bears the aboriginal name "Nehe." Elsewhere (Indig. Fl. Haw. Isls. pl. 21. 1885) Mrs. SINCLAIR illustrated a spray of Nehe, but her plate portrays an entirely different species (a form of *L. connata* Gaud. [DC.]).

LIPOCHAETA PROFUSA **robustior** Degener & Sherff var. nov.—A specie capitulis majoribus demum cum fructibus 6–8 mm. latis et 5–7 mm. altis differt.

**Specimens examined:** *Otto Swezey* (*Degener* distrib. no.) 4185, in lowlands, 2 miles from Kekaha, Isl. Kauai, Hawaiian Isls., Jul. 18, 1932 (type, Herb. Field Mus.).

LIPOCHAETA HETEROPHYLLA **molokaiensis** var. nov.—Forsitan robustior, foliis plerumque indivisis vel hastato-trilobis, siccis nunc caeruleo-viridibus nunc flavidulo-viridibus, lamina vel lobo principali rotundo ad lanceolato.

**Specimens examined:** *C. N. Forbes* 110*Mo*, Mauna Loa, western Molokai, June, 1912 (Herb. Field Mus., 2 sheets); *J. F. Rock* 10287, west end of Molokai, 1910 (type, Herb. Field Mus.: cotype, Herb. Gray; *pro specie nova a Rockio habita*).

A variety at first considered by me as a valid species and later found to have been previously so treated by ROCK, who appears from his several collections and his herbarium determinations to have given it special attention.

LIPOCHAETA HETEROPHYLLA **malvacea** Degener & Sherff, var. nov.—Fruticosa, valde divaricateque ramosa ramis ramulisque teretibus subcandidis scabrido-hispidulis vel demum nitido-subglabratis. Folia numerosa opposita alato-petiolata petiolis 5–15 mm. longis basi dilatato-connatis, petiolo adjecto 2–6 cm. longa, circumambitu ovata, plerumque 3-lobata lobis obtusis irregulariter dentatis vel quidem plus minusve sublobulatis utrinque scabrido-hispidis subtus perspicue venosis. Capitula numerosissima (unica herbarii scheda ∓100 ferente), cymose disposita pedicellis tenuibus 1–4 cm. longis, ad anthesin minima (pansa tantum circ. 6–7 mm. lata et circ. 3 mm. alta). Involucri scabrido-hispidi bracteae exteriores circ. 6, late oblongo-lanceolatae vel ovato-lanceolatae apice plus minusve acutae demum 3–4 mm. longae. Flores ligulati circ. 8, aurei, ligula obovati, apice acriter 2–3-denticulati, 3–4 mm. longi. Achaenia subbrunnea, valde verrucosa, obovato-cuneata, angulis

lateralibus alata alis saepe incisis vel interruptis apice in aristam squamelliformem incurvatam productis, corpore 2.4–3 mm. longa, exteriora superne 3–4-angulata angulis medianis plus minusve in aristam productis, interiora obcompressa omnia apicis centro plerumque tenuiter erecteque 2-aristata atque inter aristas erecte plurisetosa.

**Specimens examined:** *Otto Degener* 4199, on arid, rocky plain near Kolo, western Molokai, Hawaiian Isls., Apr. 5, 1928 (2 type sheets, Herb. Field Mus.: cotypes, Herb. Berl.; Herb. Brit. Mus.; Herb. Gray; Herb. Kew; Herb. Mo. Bot. Gard.; Herb. N.Y. Bot. Gard.; Herb. Par.).

This variety is so unique in appearance that for some time I regarded it as a distinct species. From *L. heterophylla* proper it is separated by its much more numerous heads, these being at anthesis only about 6–7 mm. wide, not 1–2.2 cm. wide as in *L. heterophylla*. The foliage when much dissected has a distinctly *malvaceous* aspect.

*L. heterophylla* and its two varieties may be identified by the following key:

Aegra, vix erecta, ramis gracilibus saepe subvolubilibus vel subscandentibus, foliis valde polymorphis, plantis lanaiensibus mauiensibusque raro molokaiensibus
*L. heterophylla* sensu stricto

Robusta, ramis validioribus nec subvolubilibus nec subscandentibus, foliis plerumque subsimplicibus vel hastato-trilobis siccis plerumque flavido-viridibus raro pedatim 3–5-lobatis, plantis molokaiensibus.....var. *molokaiensis*

Pro parte intermedia, plerumque ramosissima, foliis siccis caeruleo-viridibus saepius multo divisis, capitulis numerosissimis pansa ad anthesin plerumque sub 1 cm. latis, plantis molokaiensibus
var. *malvacea*

**Lipochaeta bryanii** sp. nov.—Suffruticosa, ramosa ramis angulatis vel etiam subalatis minute adpresso-hispidulis gracilibus. Folia numerosa, petiolata petiolis tenuibus usque ad 1.5 cm. longis, petiolo adjecto principalia circ. 4–5 cm. longa alia plerumque 2–3 cm. longa, circumambitu oblonga vel hastata, apice obtusa basi late cuneata margine leviter pauciterque crenato-dentata vel basi utrinque etiam 1-lobulata, rugulosa, densissime ac minutissime adpresso-

hispidula, subtus elevato-venosa. Capitula ad apices ramulorum disposita, tenuiter pedicellata pedicellis adpresso-hispidulis 1–4 cm. longis, radiata, pansa ad anthesin circ. 1.4–1.7 cm. lata et circ. 4–5 mm. alta. Involucri adpresso-hispiduli bracteae exteriores 4 vel 5, ovatae vel late oblongae, apice obtusae vel rotundatae, ad basim aegre gibbosae, 3.5–4.5 (raro –5) mm. longae. Flores pilosuli, ligulati plerumque 5, flavi, ligula oblongae vel anguste obovatae, apice bidenticulati, 6–8 mm. longi. Achaenia exteriora tumida orbiculata vel obovato-cuneata, 3–4-gona, valde verrucosa vel etiam verrucoso-spinulosa (praecipue ad apicis circumferentiam), angulis raro plus minusve interrupto-alata, corpore circ. 2–2.5 mm. alta, apice erecte hispida et ad centrum 2–3-aristata aristis subsquamelliformibus brevibus erectis acribus.

**Specimens examined:** *E. H. Bryan Jr.* 736, amid pili grass, on slope, Isl. Kahoolawe, Hawaiian Isls., Feb. 16, 1931 (type, Herb. Bish. Mus.).

The species is named in honor of the collector, *Edwin H. Bryan Jr.*, the Bernice Pauahi Bishop Museum in Honolulu. It was largely through his kindness that I was permitted to study this and other specimens of *Lipochaeta* belonging to that institution.

**Lipochaeta kahoolawensis** sp. nov.—Fruticosa, plus minusve ramosa ramis teretibus sulculatis adpresso-hispidulis. Folia alato-petiolata petiolis 1–2.2 cm. longis inferne late dilatato-connatis superne 2–6 mm. latis, petiolo adjecto 6–8 (raro –9) cm. longa, laminae circumambitu plus minusve deltoideo-ovata, palmatim 3–5-lobata lobis acutis vel subacutis, margine acriter irregulariterque serrata, supra adpresse scabrido-hispidula, subtus molliter ac dense pilosa. Capitula paniculato-cymose ad ramorum apices disposita (10–35-adgregata), tenuissime pedicellata pedicellis hispidis saepius 3–10 mm. longis, radiata, pansa ad anthesin 8–14 mm. lata et 4–5 mm. alta. Involucri hispidi parvi bracteae exteriores circ. 4, oblongo-lineares vel lanceolato-oblongae, apice subacutae, plerumque circ. 2 mm. longae. Flores ligulati 5–8, flavi, ligula anguste obovati, apice denticulati, circ. 6–7 mm. longi. Achaenia atra vel atro-brunnea, exteriora cuneata, 3–4-gona, faciebus minute verrucosa, angulis plus minusve (interdum interrupte) alata, corpore vix 2 mm. longa, superne plus minusve erecto-setosa, alarum apicibus in dentes erecto-

incurvatos tenues acres producta, suo apice centraliter saepius 2–3-aristata aristis brevibus.

**Specimens examined:** *Jules Remy* 269, Isl. Kohoolave (Kahoolawe), Hawaiian Isls., 1851–1855 (type, Herb. Par.).

The leaves suggest those (particularly the more definitely lobed ones) found on *L. heterophylla* var. *molokaiensis*. The inflorescence, however, is very distinct. This appears at first glance like a true panicle. The heads are numerous, minute, and in measurements for the exterior involucral bracts and exterior achenes may easily be distinguished from those of all related species.

**Lipochaeta perdita** sp. nov.—Suffruticosa, forsitan procumbens vel suberecta, ramosa ramis gracillimis rotundato-angulatis adpresso-hispidulis. Folia opposita petiolata petiolis tenuibus circ. 6–14 mm. longis, petiolo adjecto 2–4 cm. longa et 1–2.2 cm. lata, simplicia, ovato-deltoidea, apice subobtusa vel subacuta sed non acuminata, basi subtruncata vel truncata (nec vere subcordata), margine leviter sed subacriter crenato-serrata, supra subrigide subtus molliter adpresso-hispida. Capitula subsolitaria, longe tenuiterque pedunculata pedunculis adpresso-hispidis usque ad 9 cm. longis, demum (cum fructibus) circ. 12–13 mm. lata. Involucri adpresso-hispiduli bracteae exteriores 4 vel 5, ovatae vel subobovatae, apice subacutae vel subobtusae, circ. 5 mm. longae. Flores ligulati non visi. Achaenia exteriora obovato-cuneata, tumida, 3–4-gona, valde verrucosa, rubido-atra, angulis interdum plus minusve interrupto-alata, corpore 2.5–2.9 mm. longa et superne circ. 2.5 mm. crassa, apice ad circumferentiam sparsim erecto-spinulosa, aliter brevissime erecto-setosa et centraliter interdum pauciaristata aristis antrorsum hispidulis acribus.

**Specimens examined:** *David Nelson*, Hawaiian Isls., 1778–1779 (type, Herb. Brit. Mus.).

The general aspect is deceivingly like that of *L. subcordata* A. Gray. The leaves are smaller, however, than the average leaves of that species and are not so acuminate at the tips. The much larger capitula, these solitary or nearly so, are further means of distinction from *L. subcordata*. The type sheet bears five sprays, all closely similar. The species apparently has not been re-collected since the visit of NELSON to the Hawaiian Islands more than a century and a

half ago, and may well be one of those numerous endemic species which have been destroyed with the advent of civilization.[7]

**Lipochaeta venosa** sp. nov.—Subherbacea, ramosa ramis angulatis gracilibus scabrido-hispidis. Folia numerosa, tenuiter petiolata petiolis plerumque 4–10 mm. longis petiolo adjecto nunc omnia tantum 1–2 cm. longa nunc nonnulla 3–5 cm. longa, caeruleo-viridia, hastulato-trilobata, supra valde depresso- subtus valde elevato-venosa, supra sparsim breviterque hispidula subtus dense hispida lobis irregulariter crenato-dentatis. Capitula ad apicem ramulorum disposita, tenuiter pedicellata pedicellis villosis, radiata, pansa ad anthesin ∓1.3 cm. lata et circ. 5 mm. alta. Involucri villoso-hispidi bracteae exteriores circ. 5, oblongae vel late ovatae vel etiam perspicue dilatatae ac subrotundatae, apice obtusae, saepe patentes, 3–4.5 mm. longae. Flores ligulati circ. 6–8, flavi, ligula plus minusve oblongi, apice denticulati, ∓6 mm. longi. Achaenia matura minima, atra, cuneata vel obovata, inferne glabrata superne minute setulosa, angulis exalata vel superne vix cartilagineo-verrucosa, corpore 1.5–2.2 mm. longa, plerumque 5–6-aristata, aristis brevibus acribus stramineis antrorsum hispidulis.

**Specimens examined:** *J. F. Rock* 8349, Nohonaohae Crater, Waimea, Isl. Hawaii, June, 1910 (type, Herb. Field Mus.: cotype, Herb. Gray).

**Lipochaeta rockii** sp. nov.—Fruticosa, ramosa ramis teretibus vix sulculatis, hispidula. Folia tenuiter petiolata petiolis circ. 7–14 mm. longis, petiolo adjecto 3–5 cm. longa, circumambitu triangulato-ovata ad triangulato-cordata, 3–5-lobata vel subpedatim 3–5-partita, lobis membranaceis dentatis vel rursus lobulatis subrotundis ad lineari-oblongis plerumque obtusis terminali longiore vel etiam elongato ac lateraliter laciniato-dentato. Capitula subcorymbosa, tenuiter pedicellata pedicellis hispidulis plerumque 3–6 cm. longis, radiata, pansa ad anthesin 1.5–2.4 cm. lata et 5–6 mm. alta. Involucri hispiduli bracteae exteriores circ. 5 vel 6, nunc lanceolato-oblongae nunc ovatae vel obovatae sed ad apicem plus minusve acuminatae, 2–4 mm. longae. Flores ligulati circ. 8, flavidi, ligula

[7] The trivial name has been selected, however, with its twofold meaning in mind, namely, either *ruined* or *lost*.

moderate vel latissime oblongi, apice acriter bidentati, 7–9 mm. longi. Achaenia atra, faciebus minutissime adpresso-hispidula et saepe subverrucosa, exteriora 3–4-gona plus minusve alata alis interrupte erosis, omnia cuneata, corpore 2–2.8 mm. longa, apice truncato erecte setosa et ad centrum plerumque 2-aristata aristis tenuibus brevibusque.

**Specimens examined:** *Otto Degener* 4212 *pro minima parte*, dry, grassy, rocky plain near Waiahewahewa Gulch, Isl. Molokai, Apr. 18, 1928 (Herb. Field Mus.); *Abbé U. Faurie* 1001, Kamolo, Isl. Molokai, June, 1910 (Herb. Brit. Mus.; Herb. Deless., 2 sheets); *idem* 1002, *eodem loco et tempore* (Herb. Brit. Mus.; Herb. Deless.); *C. N. Forbes* 220*Mo*, mountains below Puu Kolekole, Isl. Molokai, July, 1912 (Herb. Field Mus., 3 sheets); *Wm. Hillebrand*, Isl. Oahu (Herb. Berl.; Herb. Kew); *idem*, Isl. Molokai (Herb. Berl., 2 sheets); *J. F. Rock* 6156 (type, Herb. Gray: cotype, Herb. Field Mus.).

This species and its varieties have commonly been confused with *L. lobata*.

LIPOCHAETA ROCKII **subovata** var. nov.—Folia principalia 4–7 cm. longa et usque ad 5 cm. lata, deltoideo-ovata vel subovata, grosse dentata vel inciso-lobulata, petiolis anguste alato-marginatis.

**Specimens examined:** *Jules Remy* 270, Isl. Molokai, 1851–1855 (type, Herb. Par.).

LIPOCHAETA ROCKII **dissecta** var. nov.—Folia marginato-petiolata, principalia 5–7.5 cm. longa, plerumque 3–5-partita lobis lineari-oblongis acriter inciso-dentatis vel pinnatifidis.

**Specimens examined:** *Wm. Hillebrand*, western Maui (Herb. Berl.); *idem* 27, Isl. Maui (Herb. Gray; Herb. Kew; *cum L. heterophylla* A. Gr. *commixta*); *U.S. Exploring Expedition under Capt. Wilkes*, eastern Maui (type, Herb. U.S. Nat.: cotype, Herb. Gray; *cum L. heterophylla* A. Gr. *commixta*); *eadem, sine loco sed sine dubio* western Maui (Herb. N.Y. Bot. Gard.); *eadem*, western Maui (Herb. U.S. Nat.; *cum L. heterophylla* A. Gr. *commixta*).

The specimens from western Maui have the leaf lobes less finely dissected but are hardly to be separated varietally.

*L. rockii* and its varieties may be separated according to the following key:

a. Folia plerumque 3–5-partita
  b. Foliorum lobi lineari-oblongi acriter inciso-dentati vel pinnatifidi, planta mauiensi.....var. *dissecta*
  b. Foliorum lobi latiores moderate dentati rare paulo incisi, planta molokaiensi et oahuensi.....*L. rockii* sensu stricto
a. Folia grosse dentata vel inciso-lobulata, planta molokaiensi var. *subovata*

**Lipochaeta intermedia** Degener & Sherff, sp. nov.—Fruticosa, foliosa, ramosa, gracilis. Folia opposita petiolata petiolis tenuibus 8–14 mm. longis, petiolo adjecto 3–5.5 cm. longa, simplicia, minute acriterque serrata, deltoideo-lanceolata, basi late cuneata vel rotundata, apice moderate acuminata, crassiuscula, utrinque incano-hispida, subtus valde elevato-venosa. Capitula numerosa corymboso-cymosa, gracillime pedicellata pedicellis usque ad 2 cm. longis, radiata, pansa ad anthesin 1.2–1.6 cm. lata et circ. 5–6 mm. alta. Involucri hispidi bracteae exteriores circ. 4 vel 5, nunc oblongo-lanceolatae nunc ovatae, apice saepius subacutae, ∓4 mm. longae. Flores ligulati plerumque 5, flavi, ligula obovato-oblongi, apice subintegri, tantum circ. 6–7 mm. longi. Achaenia atra, cuneata, circ. 2.3–2.5 mm. longa, exteriora 3–4-gona, saepe tumida, glabra vel angulis obsolete muriculata, supra saepe spinosa et centraliter 1–6-aristata aristis brevibus tenuibus antrorsum hispidulis.

**Specimens examined:** *Alfred Meebold* (*Degener* distrib. no.) 4254, among lava, Huehue, Kona District, Isl. Hawaii, Hawaiian Isls., May, 1932 (type, Herb. Field Mus.).

A species somewhat intermediate between *L. lavarum* (Gaud.) DC. (especially the var. *ovata*) and *L. subcordata* A. Gray. From *L. lavarum* it differs at once in its smaller and much more numerous capitula; from *L. subcordata* it is easily distinguished by its more slender and basally wide-cuneate or rounded (not subcordate or cordate) leaves, etc.

**Lipochaeta tenuis** Degener & Sherff, sp. nov.—Herbacea vel vix suffruticosa, caulibus plus minusve procumbentibus gracillimis elongatissimis angulatis glabratis vel sparsim adpresso-hispidulis ∓1 m. longis. Folia tenuiter petiolata petiolis circ. 1–1.5 cm. longis petiolo adjecto circ. 3.5–5.5 cm. longa, indivisa, tenera, deltoideo-lanceolata vel parce deltoideo-ovata, subtus pallida, utrinque minutissime ad-

presso-hispidula, marginibus acriter serrata circ. 7–9 dentibus pro unico latere, apice acuto mucronata. Capitula perpauca, tenuiter pedunculata pedunculo ∓3 cm. longo, radiata, pansa ad anthesin ∓2.6 cm. lata et circ. 6 mm. alta. Involucri pallidi bracteae exteriores 4 vel 5 oblongo-ovatae vel oblongo-lanceolatae apice acutae ∓6 mm. longae. Flores ligulati ∓10, intense aurei, ligula linearioblongi, apice interdum denticulati, circ. 1.1–1.3 cm. longi. Achaenia primum aristata demum (unicum maturum exterius visum) apice calva, cuneato-obovata, brunnea, trigona, obscure verrucosa, angulis aegre vel obsolete interrupto-alata, apice minutissime setulosa, circ. 2.4 mm. longa.

**Specimens examined:** *Otto Degener*, a long, straggling, suffrutescent herb in the rain forest, Waianae Valley, up toward Mt. Kaala, Isl. Oahu, Apr. 24, 1932 (type, Herb. Field Mus., 2 sheets: cotype, Herb. Kew).

CHICAGO NORMAL COLLEGE

# STRUCTURE AND DEVELOPMENT OF PILULARIA MINUTA. DURIEU MANUSCRIPT[1]

DUNCAN S. JOHNSON

(WITH FORTY-FOUR FIGURES)

## Introduction

This diminutive species of the Marsileaceae, first discovered by DE NOTARIS in 1835 at Pula in Sardinia, has since that time been collected at two other stations in that island, in Algeria, in southern France, and in Smyrna (**4, 7**). Although thus rather widely distributed about the Mediterranean it has, perhaps as a consequence of its small size and its delicacy, only rarely been collected, its structure has been but little studied, and its development has not been described at all.

Because the capsule of *Pilularia minuta* has but two sori, the smallest number known in the whole family, the writer has long wished to compare its development and detailed structure with those of the capsules of *P. globulifera* and *Marsilea quadrifolia* (JOHNSON **10, 11**). The first opportunity to do this came recently, when herbarium specimens of this plant collected by DURIEU in Algeria were received on loan from the National Herbarium, the New York Botanical Garden, and the University of Montpellier, and when fruiting plants preserved in alcohol were received from Professor J. PAVILLARD of Montpellier. Grateful acknowledgment is here made to the institutions mentioned, and above all to Professor PAVILLARD for the valuable alcoholic specimens of the plant.

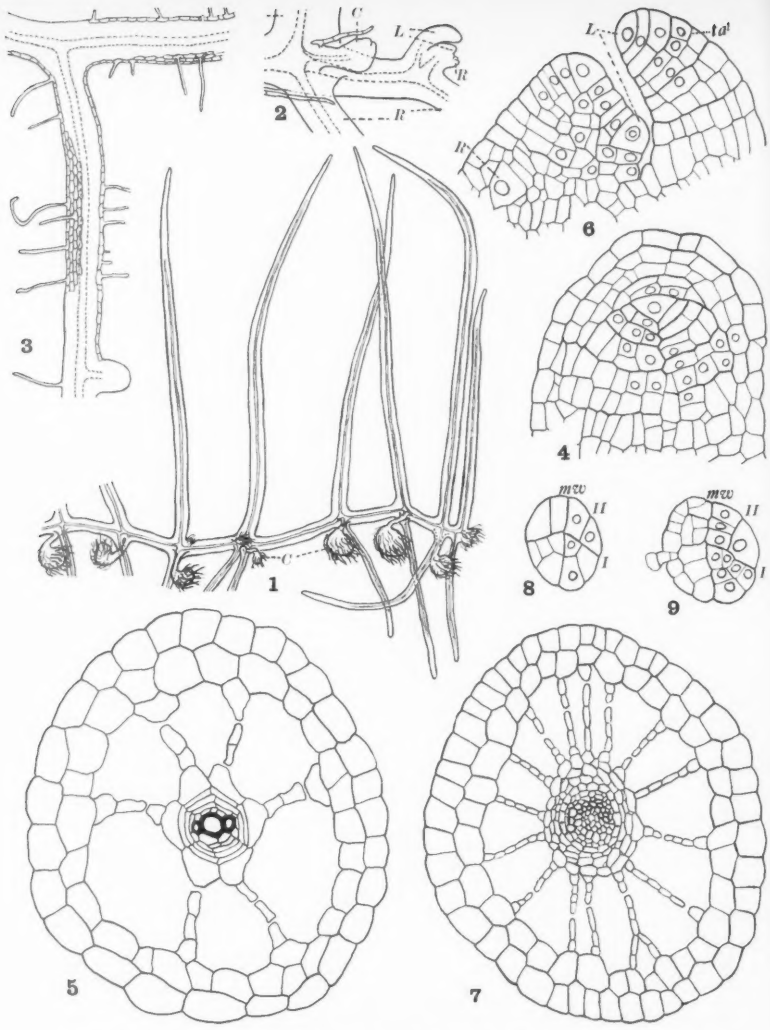
From the study of whole shoots mounted in glycerin, of hand sections of capsules, and especially of paraffin sections of all available alcoholic material of the stem, leaf, and sporocarp, many of the essential features of the later development of these organs have been determined. Unfortunately the very earliest stages of development of the sporocarp could not be found.

[1] Botanical contribution from the Johns Hopkins University, no. 117.

**Vegetative structure**

The stem of this most delicate of all the Marsileaceae is approximately 4–6 cm. long, 0.5–0.7 mm. in diameter, and consists of twelve or more segments. Each node of the stem bears a single leaf, one or two roots, often a bud or branch, and, when fruiting, a single sporocarp (figs. 1, 2). The individual internodes of the mature parts of the stem attained a length of from 3 to 6 or 8 mm. The internodes of the stem had an average thickness of 500–700 μ. The stele of the internode is less than one-fifth the diameter of the stem itself. The initial of the stem is probably three-sided although this was not determined with certainty (fig. 6). The younger parts of the stem are well protected by very numerous trichomes. The adventive roots of this species are oftenest simple, but in certain cases there are branches of the second and third orders. The longest simple root seen was 20 mm. in length, and the primary branch shown in figure 3 was 16 mm. long. Root hairs were abundant (fig. 3) although the roots seemed entirely free of solid soil particles. The root initials arise near the stem apex, approximately opposite (that is, below) those of the leaves. The root initial is tetrahedral (fig. 4) and gives rise to a well-marked root cap in addition to the tissues of the root proper. The average diameter of the root is about 150 μ, and that of its vascular bundle about 30 μ.

The leaf is often 35–40 mm. in length and has a diameter of 500–700 μ. Its vascular bundle is little more than 100 μ in diameter. The leaf arises from a single, evidently two-sided initial which is formed on the upper surface of the stem, apparently to the right or left of the mid-line (fig. 6). The total number of segments finally cut off by this initial could not be traced definitely, as was done in the case of *Marsilea* (**10**). Approximately transverse sections near the tip of the leaf show, however, that the segments of the initial are practically semicircular discs (fig. 6), just as they are known to be in *Marsilea* and *Pilularia*. Moreover, the earlier longitudinal anticlinal walls dividing these segments, the "section walls" of JOHNSON (**10, 11**) have precisely the position that they were shown to have in *Marsilea quadrifolia* and *Pilularia globulifera* (figs. 8, 9). Cross-sections of the mature leaf seem to show clearly that the epidermis, mesophyll, and vascular bundle are differentiated much as they are




FIGS. 1–9.*—Fig. 1, lateral view of growing end of fruiting shoot, showing stem, leaves, and roots with 7 sporocarps, the largest 3 almost mature. ×4. Fig. 2, 2 segments of similar shoots, showing 2 leaf rudiments and one very young sporocarp. ×28. Fig. 3, main root with primary and secondary branches, vascular system, and root hairs. ×42. Fig. 4, longitudinal section of young root apex showing initial and root cap. ×350. Fig. 5, transverse section of mature root showing 6 air canals, 6 endodermal cells, and very simple vascular strand. ×350. Fig. 6, longitudinal section (approximately sagittal) of stem apex showing initial of stem, those of 2 young leaves, and root initial. ×350. Fig. 7, transverse section of nearly mature stem showing 15 air canals and structure of stele. ×180. Fig. 8, approximately transverse section of tip of young leaf showing semicircular form of segments of leaf initial and (at I and II on right) positions of first longitudinal anticlines dividing this segment. ×350. Fig. 9, similar section of leaf just older than that in fig. 8, showing (at right) earlier longitudinal walls in one segment of leaf initial. ×350.

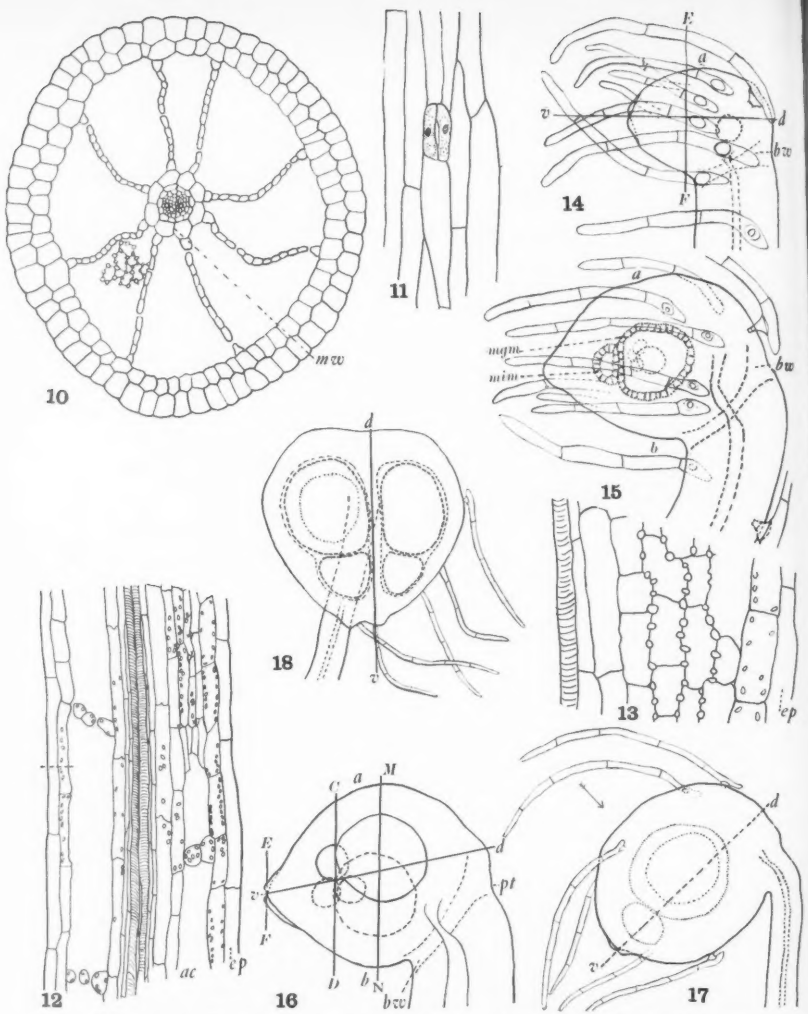
* Abbreviations: *a*, apex; *abax*, abaxial side of bundle; *ac*, air canal; *adax*, adaxial side of bundle; *b*, basal side of capsule; *bw*, basal wall; *C*, capsule of sporocarp; *d*, dorsal; *ep*, epidermis; *id*, indusium; *L*, leaf; *mast*, macrostoma; *mb*, meridional bundle; *mc*, marginal cell; *mgm*, megasporangium; *mgp*, megaspore; *msm*, microsporangium; *mw*, median wall; *pab*, placental bundle; *pt*, pit; *R*, root; *S*, stem; *sc*, coral canal or cavity; *st*, stoma; *la*$^1$, first transverse anticline; *tc*, trichome; *v*, ventral; *I*, *II*, first and second section walls.

Figs. 1–3 and 14–18 are surface views of the structures shown; all other figures are from microtome sections of material fixed in alcohol, imbedded in paraffin.

in *Marsilea*. Early in its development the leaf grows more rapidly on the dorsal side, and in consequence pushes around in front of the stem growing point. It later continues this differential growth to form the characteristic watchspring-like, circinately coiled leaf. A cross-section in the mature leaf shows that the epidermis is supported by a well developed hypodermis beneath which lie eight relatively large, longitudinal air canals separated by perforate radial partitions (figs. 10, 12). The minute vascular bundle consists of relatively few vascular elements, usually but two tracheae, and is surrounded by a weakly developed endodermis. The trichomes are deciduous, so that the mature leaf is naked except for the remnants of the second cell of the trichome which sometimes adheres to the persistent basal cell that lies imbedded between the normal epidermal cells. The stomata of the leaf are relatively few. The guard cells are elongated (with the leaf) to four or five times their transverse dimension (fig. 11).

**Sporocarp**

The sporocarps of *Pilularia minuta* are attached to the stem, a single sporocarp in front of the base of each leaf. The relatively elongated but slender peduncle of the mature sporocarp may be 1.5–2.5 mm. in length. In general young sporocarps are those nearer the stem apex, although some of those found farther from the apex are smaller, probably because retarded in development. The sporocarp even at maturity is densely covered with glistening, tawny hairs, so that it is not always easy to determine its exact external form (figs. 1, 2, 14). The capsule of the sporocarp is more or less ovoid, often decidedly flattened on the side toward the peduncle (figs. 23, 27, 31), and the "tip" (really ventral side) of the capsule when mature is bent downward against the base or middle portion of its own peduncle (figs. 18, 21, 22). This flattening of the capsule, together with the bending of its stalk, makes it externally a distinctly dorsiventral or bilaterally symmetrical structure, a type of symmetry which is based on its mode of development and is carried out also in every detail of internal structure, as will be described later. The tip of the capsule, even when practically mature, shows two papillae, the origin of which also will be mentioned later. At the base of the capsule (that is, at the point of juncture with the peduncle



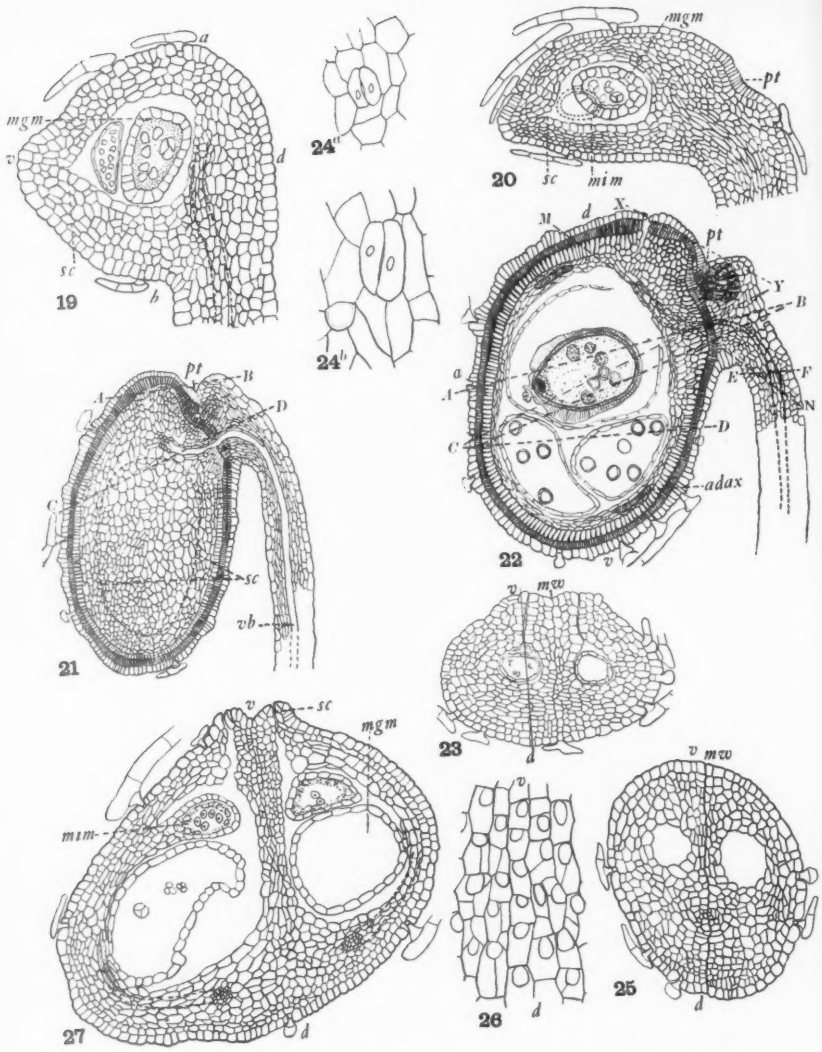

FIGS. 10–18.—Fig. 10, transverse section of mature leaf, near middle, showing vascular bundle, endodermis, 8 air canals, 8 perforated partitions, and continuous hypodermis and epidermis. ×120. Fig. 11, tangential section at surface of mature leaf showing epidermis and stoma. ×260. Fig. 12, radial section of nearly mature leaf showing vascular bundle and perforate radial and transverse partitions between air canals. ×180. Fig. 13, portion of perforate radial partition of leaf showing form of component cells. ×260. Fig. 14, left side of young sporocarp showing relatively large trichomes at this stage, form of capsule, position on peduncle, vascular bundle of latter, and basal wall between capsule and peduncle (*a*, morphological apex of capsule). ×90. Fig. 15, similar view of somewhat older capsule showing position of megasporangium and microsporangium and vascular bundle penetrating basal wall. ×90. Fig. 16, diagram of left side of capsule older than that in fig. 15, indicating sporangia of both locules and 2 papillae marking ventral ends of soral canals (*CD*, *EF*, *MN*, *vd*, planes of sections shown in several following figures). ×90. Fig. 17, left side of almost mature capsule showing double beak, dorsal pit, and tubercle of capsule. ×30. Fig. 18, approximately apical view of same capsule (arrow in fig. 17 indicates direction) showing tip of capsule and position of megasporangium and of one of the two microsporangia in each half of sorus. ×28.

and on the abaxial side) there is a distinct depression in its surface, the so-called basal (dorsal) pit; while just basal to this at the upper end of the peduncle is a marked elevation or tubercle, probably the equivalent of the tooth on the sporocarp of *P. globulifera*.

The internal structure of the sporocarp of *Pilularia minuta* is the simplest found in any of the Marsileaceae. Whether it is a primitively simple structure or not is a question that can better be taken up later, after its development has been discussed. It may be stated here that the general plan of structure of this capsule is the same as that found in *P. globulifera*, and therefore it also is regarded as a modification of the type found in *Marsilea*.

The capsule of *P. minuta*, as is clear from examination of transparent older sporocarps, of sections (figs. 18, 27, 28, 30), or of a burst capsule, consists of two locules with one sorus each. These locules lie one on each side of the sagittal plane (that is, of the median wall) that results from the repeated segmentation of the evidently two-sided initial of the sporocarp (figs. 23, 25, 27, 29, 35). Each locule has a somewhat ovoid hemispherical shell, the outer convex side of which is formed by one-half of the 6- to 8-layered outer wall of the capsule. The flat, diametric partition wall of the locule, the indusium, is composed of three to five layers of cells that arise in each half of the capsule next to the median plane. Hence the soral cavity in each locule is separated from its fellow of the other half of the capsule by the two plane walls, each three to five cells in thickness (figs. 21, 23, 28).

Each locule (or half) of the capsule bears a single placenta, which forms a small mound of thin-walled cells projecting into the soral cavity from about the middle of the outer wall of each half of the capsule (figs. 28, 29). Each placenta is supplied by the middle one of three meridional vascular bundles that are formed in each half of the capsule. From each placenta are developed a single megasporangium, which lies near the dorsal side (or "base") of the capsule (figs. 15, 16, 18, 22, 28) and two microsporangia, which lie nearer the tip, or ventral side, of the capsule (figs. 15, 18, 22, 28). The megasporangium is about 500 $\mu$ in diameter and has a wall which at maturity is but one cell thick (figs. 19, 27, 29). The walls of the tapetal cells have by now broken down, and the contents persist, if




FIGS. 19–27.—Fig. 19, nearly sagittal section of young sporocarp (about in plane *vd*, fig. 23) showing microsporangium and megasporangium of one sorus and course (dotted) of vascular bundle from peduncle. ×156. Fig. 20, approximately sagittal (but not median) section of slightly older sporocarp showing beginning of dorsal pit and

at all, as a plasmodium-like layer about the single functional megaspore and the numerous degenerating megaspore tetrads. The microsporangia are nearly half the diameter of the megasporangium and likewise have a single layered wall (figs. 27, 28). Both microsporangia of a sorus lie at the same level and both are directed somewhat toward the tip or ventral beak of the capsule (figs. 18, 27, 28).

The single megaspore of each locule when mature nearly fills the sporangium. The many tetrads of degenerate megaspores lie about the functional one (figs. 15, 27). The megaspores are elongated, with a distinct beak but no constriction (figs. 22, 30). They average 506 $\mu$ in length and 430 $\mu$ in diameter. The wall of the megaspore is 3-layered and the wall, especially the outer layer, swells in water or potash until the diameter of the whole may reach 900 $\mu$. The megaspore when mature contains but a single nucleus, which is large and often flattened until its diameter transverse to the spore is several times its longitudinal one (fig. 22). The cytoplasm of the whole basal portion of the spore cavity is almost completely filled with large starch grains, whose diameter is almost equal to that of the microspores of this species.

Each microsporangium contains about 128 globular microspores, each of which is about 55 $\mu$ in diameter (figs. 30, 31). The wall of

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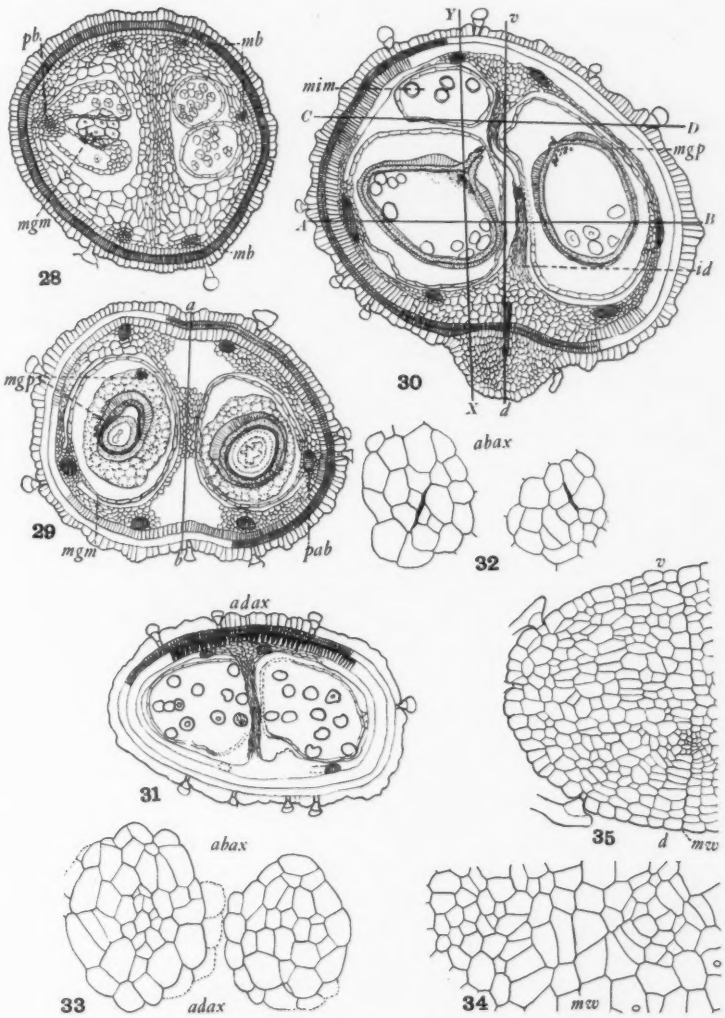
tubercle below this. ×120. Fig. 21, sagittal and median section (plane *dv*, figs. 18, 29, 30) of half-grown capsule showing outline of capsule, dorsal pit, tubercle, basal wall, bundle from peduncle, and the 3 specialized layers of wall of capsule. Note overlap in layers of basal wall just within pit; this median section passes through whole width of indusium between the 2 locules (cf. figs. 28, 39, 42). ×60. Fig. 22, approximately sagittal section of mature capsule (plane *YX*, fig. 30) showing tubercle, pit, overlapping in basal wall, course of bundle penetrating basal wall, and oblique sections of soral cavity and of 2 meridional branches of vascular bundle. ×60. Fig. 23, transverse section (plane *vd*, figs. 14, 16) of capsule about age of that shown in fig. 19 showing soral cavities still small, 2 soral canals, opening right and left of median wall (formed by segmentation of 2-faced initial of bilaterally symmetrical capsule (*cf. mw*, fig. 8 of leaf). ×180. Fig. 24, microstoma and megastoma of wall of capsule. ×350. Fig. 25, section (plane *CD*, fig. 21) through basal part of half-grown capsule; median wall very distinct. ×180. Fig. 26, part of tangential section of capsule in last figure showing epidermal cells and apical position in each of these of the rounded trichome-bearing cell formed by it. ×350. Fig. 27, nearly transverse section (longitudinal to locules and in plane *vd*, fig. 17) of half-matured capsule showing ventral papillae with soral canals, both megasporangia of capsule, and one of the two microsporangia of each locule. Dotted lines show course of short, middle bundle in each locule. ×120.

the microspore is of three distinct layers, the outer and thickest swelling greatly when soaked in water (or potash). The ripe microspore contains a single nucleus, and but few and small starch grains (figs. 22, 31).

The single vascular bundle of the peduncle is 33 $\mu$ in diameter, and consists of 35–40 cells in cross-section within the endodermis. There is no sclerenchyma strand accompanying the bundle here as in *P. globulifera* (**16,** figs. 11, 12; **11,** figs. 32, 33). This single bundle continues to be the same diameter up the middle of the peduncle to the proximal (dorsal) end of the capsule. There it bends sharply, constricts to half its former diameter, and penetrates at a right angle the thickened hypodermis or light-line layer of the basal wall of the capsule (figs. 15, 16, 21, 22). Soon after entering the capsule (figs. 22, 30) the bundle expands to its former diameter and then forks T-like to two arms which extend transversely, one into the right half of the capsule and the other into the left. Each arm after reaching a length of 60–70 $\mu$ divides to form an abaxial branch and an adaxial one (fig. 37). The former remains undivided and runs near the midplane and just within the wall of the capsule, ending near the tip of the soral canal of its locule. It thus forms one of the three meridional bundles found in each half of the capsule (figs. 22, 28, 29, 30). The adaxial branch on the contrary soon divides into two. One of these forms an adaxial meridional bundle which runs, near the median plane of the capsule on the side next the peduncle, around to the tip or beak of the capsule (figs. 22, 28, 30, 31 *adax*). The other division of the adaxial branch takes a meridional course near the middle of the locule. When it is half way to the tip, however, it turns inward to enter the placenta (figs. 27, 28, 29), and no portion of this bundle is left to run to the tip of the capsule as in *P. globulifera* (fig. 31). This feature sharply distinguishes the vascular bundle system of this species from that of *P. globulifera* and of many species of *Marsilea*, in which bundles of the same locule of the capsule fuse near the ventral side of the capsule (**4,** figs. 1–7; **10,** fig. 44; **16,** figs. 28, 29; **11,** figs. 33, 38). The vascular bundle system of this species is then decidedly simpler than that of *P. globulifera* and far simpler than that of any species of *Marsilea*.

The wall of the capsule in *Pilularia minuta* consists, as it does in




FIGS. 28–35.—Fig. 28, nearly horizontal section (longitudinal but perpendicular to median plane) (at level *CD*, fig. 16) of slightly older capsule showing rounded outline, thick median indusium, 2 microsporangia in each sorus, and tip of one megasporangium (note 3 meridional bundles of each locule, middle one ending in placenta, as shown

*P. globulifera*, of three outer layers of markedly specialized cells within which are three to six layers, or in some parts still more, of parenchymatous cells, among which are imbedded the vascular bundles. Certain of these parenchyma cells, chiefly those situated near the median plane (figs. 28, 29), finally swell to help burst the capsule and expel the spores. In two other regions (one at the middle of each valve of the capsule) it is the parenchyma layer of the wall of the capsule that becomes thickened to form the placenta (figs. 28, 29 *pab*).

Of the three more specialized layers of the wall proper of the capsule, the outer, the epidermis, consists of ordinary epidermal cells, of trichome-forming cells, and of the guard cells of the relatively few microstomata and megastomata found chiefly near the base of the capsule (figs. 24, 38). The epidermal cells are more or less polyhedral in outline with straight or wavy sides (fig. 26). Those forming the somewhat netlike elevations of the surface (18) are often 35 $\mu$ thick (perpendicular to the surface of the capsule), while the cells filling the meshes may be but 10–12 $\mu$ thick (figs. 22, 30). The epidermal cells below the bend of the peduncle are also strikingly elongated (figs. 21, 22).

The trichome-forming cells are numerous and are distributed with great regularity. Thus on the apical surface of the young capsule there is a circular trichome-forming cell cut out from the same (ventral) end of practically every surface cell (fig. 26). Later the remainder of the surface cell is cut by longitudinal and transverse

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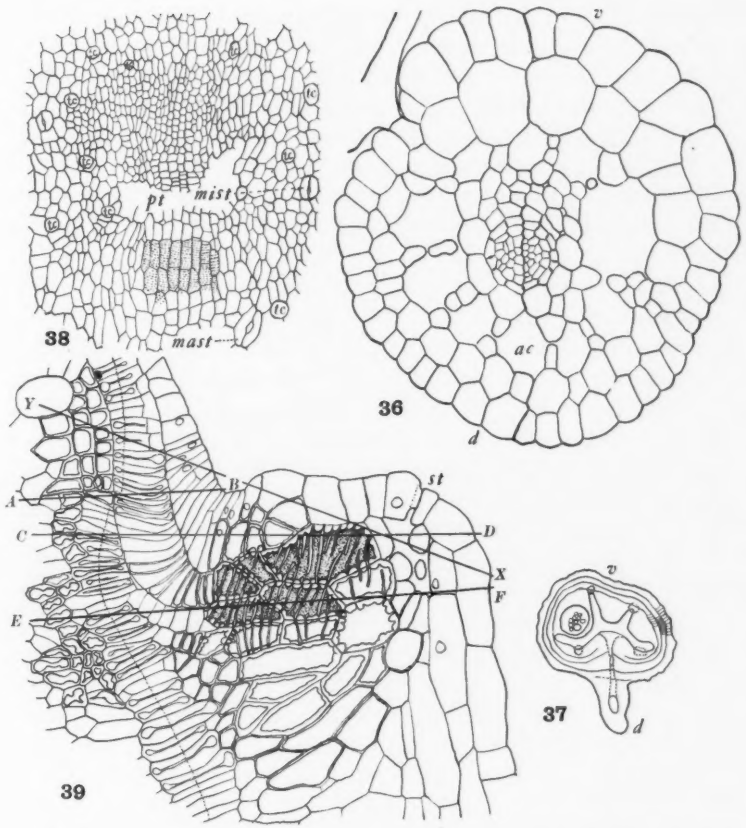
at left). ×60. Fig. 29, horizontal section of same sporocarp taken nearer the base (line *MN*, fig. 16 or *AB*, fig. 30) and thus passing through the single megaspore of each locule. Note meridional bundles, placenta, stalk, and walls of megasporangia, and (at left) plasmodium-like tapetal residue surrounding 3 degenerating megaspores and one functional megaspore. ×60. Fig. 30, obliquely horizontal section (along line *V-C*, fig. 22) of mature capsule showing 2 ripe megaspores, each inclosed by its sporangium wall, and vascular bundle penetrating basal wall. ×60. Fig. 31, approximately horizontal section of capsule near tip (plane *CD*, figs. 22 and 30) showing microsporangia only and absence, beyond placenta, of middle meridional bundle of each locule. ×60. Figs. 32–34, 3 successive horizontal sections (transverse to soral canals) through papillae of ventral tip of half-grown capsule. Soral canals closed, indication of a canal at the center of each papilla in fig. 32; median wall marking sagittal plane shown in fig. 34 at *mw*. ×350. Fig. 35, one-half of transverse section near middle of young peduncle with vascular bundle just differentiating; median wall clear. ×350.




FIGS. 36–39.—Fig. 36, transverse section of older peduncle, immediately below capsule, showing median wall, 8 air canals, and vascular bundle with well marked endodermis. ×350. Fig. 37, obliquely horizontal section (plane *MN*, fig. 22) through base of capsule showing bilateral forking of vascular bundle after entering capsule and subsequent divisions of each fork to form (as on left) 3 meridional bundles in each locule. ×30. Fig. 38, tangential section (on line *XY*, figs. 22 and 39) of pit at base of capsule showing outline of pit, character of cells of tubercle and of basal wall as cut in this plane; also distribution of trichomes and of guard cells of 3 microstomata and of one megastoma. ×180. Fig. 39, basal (dorsal) portion of median sagittal section of mature capsule showing structure of tubercle, lining of basal pit, and overlap of hypodermis in basal wall. ×350.

anticlines (fig. 26) to form four or more of the epidermal cells of the mature capsule (*cf.* **10,** figs. 2, 3, 15, 16).

The mature trichome consists of some six or seven cells altogether. The innermost is the basal cell which is about half the height of the surrounding epidermal cells and has a rather thick wall (figs. 15, 17, 22, 30). Outside of this is the funnel-shaped stalk cell on the outer end of which is borne the basal cell of the 4- or 5-celled, ventrally directed, tapering limb of the trichome (figs. 14, 17, 20). The slightly thickened but firm cell walls of the limb are pale brown and give this color (somewhat lightened by the overlapping of these nearly transparent hairs) to the ripe, dry capsule itself. The form and character of the walls of the components of the two distinct layers of palisade-like cells next below the epidermis have been but briefly described from this species by RUSSOW (**17, 18**). A fuller discussion of these layers in *P. globulifera* is given by METTENIUS (**15**), RUSSOW, and especially by MEUNIER (**16**). The outer of these two prismatic layers, the hypodermis or light-line layer, is in *P. minuta* faintly yellowish in natural color, and stains a pale blue with haematoxylin. Over most of the capsule this layer is 15–20 $\mu$ thick; that is, the component cells are 15–20 $\mu$ long (figs. 22, 30). In a considerable region above the pit, at the upper end of the peduncle, however, these cells may become 35–40 $\mu$ long (figs. 22, 39). Certain peculiarities of this layer in the basal part of the capsule will be mentioned later. The inner of the two prismatic layers of the wall, the subhypodermal layer, is composed of cells of much less regular form and size. Their radial length ranges from 30 to 40 $\mu$ and their diameter is half this or less. On the flanks of the capsule these cells are rather regularly prismatic, but toward the base of the capsule, especially at and above the point of penetration of the vascular bundle into the capsule, the single layer of these brown prismatic cells is replaced by several irregular layers of similarly thickened brown cells which here become packed together promiscuously (figs. 22, 30, 39).

There are several strikingly specialized layers or groups of tissues in the dorsal (basal) portion of the wall of the capsule, near the median plane and just within the tubercle. These structures are important not only because they furnish further evidence of the complete zygomorphic symmetry of this capsule, but also because they

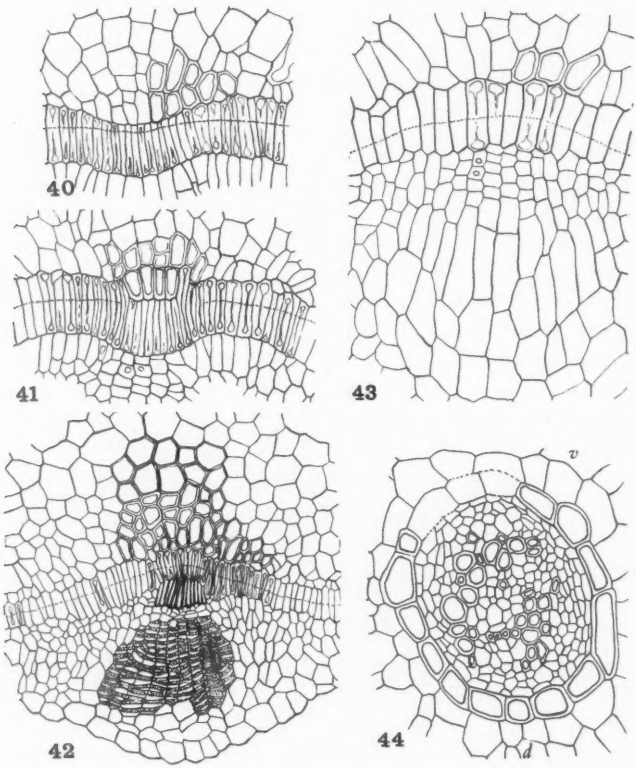
show its similarity, even in these minor details, to that of *Marsilea*. These specialized structures are as follows:

1. There is a close overlapping of a distinct median flap of the outer prismatic layer (hypodermis) as it comes down from the upper side of the capsule, upon a narrow, attenuated tongue, the same prismatic layer which here forms a firm wall across the dorsal end of the capsule (figs. 21, 22, 39, 41, 42). As will be seen from these figures, this flap is only eight or ten cells wide, and the more refractive zone which usually forms the light-line across the middle of each hypodermal cell has here, at the overlap, moved toward the inner ends of these cells, finally to disappear before quite reaching their inner ends (figs. 39, 40, 41). These cells of the outer overlapping layer are continued, beyond where the light-line disappears, by a layer of prismatic cells which are still more elongated but are thin-walled and distinctly curved. This layer finally bends outward to abut upon the hypodermal or subhypodermal layer of thickened cells of the tubercle (figs. 21, 39). The tongue of hypodermal cells that runs up from below to underlap the flap from above may be only five or six cells wide (fig. 41), and a similar shift of the light-line occurs here. In this case, however, it is toward the outer ends of the cells (figs. 22, 39, 42). The cells of this tongue also, beyond the middle of the overlap, are thinner-walled; but instead of becoming longer radially they divide to form two layers of cells, each a half or a third the usual thickness, and finally the inner of these layers abuts upon a layer of thick-walled, brownish, subhypodermal cells (fig. 39).

2. Another peculiar tissue found at the base of the capsule is a considerable group of thin-walled, radially elongated, epidermal cells that forms the bottom of the pit, just above the tooth (figs. 19, 38). These differ markedly from the shorter, brown-walled cells composing the rest of the epidermis. They gradually decrease in length and, above, they finally abut upon a single layer of epidermal cells, while below they are replaced by two layers of similarly thin-walled, colorless cells just above the tubercle (fig. 39).

3. In the basal "tooth" or tubercle of the capsule occurs a third type of specialized cell, namely, the radially elongated scalariform, thickened cell. This forms a group about 8–10 cells in width and 6–8 cells in vertical depth (figs. 22, 39, 42). The character of the

thickenings on the walls of these cells suggests that they may be water-storage tracheids or perhaps have mucilaginous walls which at germination swell to enlarge this part of the capsule and thus




FIGS. 40–44.—Fig. 40, portion of obliquely horizontal section (perpendicular to basal wall) just above line *AB* in fig. 39 showing outer overlapping layer of thickened cells as continuous around whole base of capsule. ×180. Fig. 41, portion of similar section but at level *AB* of fig. 39 and so showing overlap of two layers of hypodermis in basal wall. ×350. Fig. 42, portion of similar section, but near level *CD* in fig. 39, showing tubercle and inner (lower) wall continuous at overlap. ×180. Fig. 43, portion of section like last but at level *EF* in fig. 39, showing continuous single basal wall below overlap. ×350. Fig. 44, transverse section of stele of mature stem showing endodermis, vascular elements, and radial partitions of cortex. ×350.

permit water to enter the capsule itself and so to aid in opening the latter.

4. The characteristic aerating tissues of the capsule are far more abundant in the outer part of the tubercle itself and just ventral to the basal pit than elsewhere. Stomata are present about (and especially above) the pit, and they open into deep air canals that penetrate through both prismatic layers to the relatively abundant intercellular spaces between the underlying parenchyma cells (fig. 22). There are some stomata also on the dorsal side of the peduncle which open into the longitudinal air canals in this stalk and thus connect with the intercellular spaces of the tubercle itself (fig. 22).

All of the four groups of tissues just described are located on or near the dorsal line of the capsule or peduncle. In other words, they all lie in or near the plane of symmetry and so all serve further to emphasize the zygomorphic character of the sporocarp, which is, as previously seen, already clear from the arrangement of the vascular bundles, the soral canals, the indusia, and the sporangia.

Concerning the functions of the structures found at the base of the capsule, it is seen from what has been said that both layers of hypodermal cells, at the overlap of these in the base of the capsule, are decidedly thinner-walled than they are elsewhere. Since the neighboring cells of the epidermis (fig. 39) are likewise unthickened, it is clear that this is the one area of the whole wall of the capsule most readily permeable to liquids. The presence of air spaces among the cells of the tubercle, and of others connected with the stomata above the dorsal pit, indicate that this portion of the wall of the capsule is also the one through which gas interchange with the atmosphere can most readily occur. It also seems possible, from the readiness with which the cells of the two overlapping layers separate along the plane of contact, that gases may, under certain conditions of distention or shrinkage of the surrounding tissues, actually pass through this intercellular slit to or from the capsule proper.

### Development of capsule

Unfortunately it has not proved possible to find the single initial of a sporocarp at the base of the leaf such as was demonstrated in the case of *Marsilea quadrifolia* (10, figs. 22, 23). Nor was it possible, as

in the other Marsileaceae studied (**10, 11**), to secure longitudinal and horizontal sections of a sporocarp of *P. minuta* young enough to show its continuing growth by a single bifacial apical cell (*cf.* **10,** figs. 34, 38 and **11,** figs. 13, 14). But the mode of growth of the leaf of *P. minuta* is clearly identical with that demonstrated in *Marsilea* and *P. globulifera* (*cf.* figs. 5, 6 with **10,** figs. 2, 6, 7 and **11,** figs. 3, 4, 5). Moreover the cell arrangement in cross-sections of the youngest obtainable capsules of *P. minuta* (figs. 23, 25, 35) corresponds closely with similar sections of young capsules of the other two Marsileaceae mentioned (**10,** figs. 32, 33 and **11,** figs. 19, 24, 26). It therefore becomes practically certain that the earlier steps in the development of the sporocarp of *P. minuta* must correspond essentially with these same stages of *P. globulifera* and so with those in *Marsilea quadrifolia*.

Thus cross-sections of the young capsules of *P. minuta* show a diametric median wall, identical in position, and probably in origin, with the median wall found in capsules of *P. globulifera* (figs. 23, 25). This median wall, which in *P. globulifera* was shown to mark the line of juncture of the successive segments cut from the right and left faces of the bifacial initial, is seen clearly not only in cross-sections of the fertile portion or capsule of the sporocarp of *P. minuta*, but it also stands out distinctly in cross-sections of the peduncle (figs. 35, 36), and even in horizontal sections of the sterile tip, or of the base of the capsule (figs. 25, 34). This median wall evidently results from segmentation of a 2-sided initial, which is obviously the developmental foundation for the bilateral or zygomorphic symmetry of this sporocarp. The median wall lies in the plane of symmetry (the sagittal plane) which passes vertically between the two sori in a face view of the capsule (fig. 18); in a horizontal or cross-section it passes vertically through the indusium that separates the two soral cavities (figs. 23, 25, 27, 29). A median sagittal section of the capsule will not touch either soral cavity, therefore, but will cut through the indusium for its whole length (figs. 21, 28).

Further evidence of the bilateral symmetry of this capsule is seen in the pair of soral canals and the two ventral papillae usually marking the openings of these canals in half-grown capsules (figs. 14, 16, 17, 18, 27, 33). Comparison of such figures with figures of the same

stages in *P. globulifera* (**11**, figs. 19, 24, 25, 26) leaves no doubt that the six sporangia of the two sori of *P. minuta* have arisen, as was demonstrated for the more numerous sporangia of the four sori of *P. globulifera*, from originally superficial marginal cells of the ventral side that have been secondarily buried by the more vigorous growth, ventralward, of the surrounding cells, the cells that are to form the indusium and the wall of the capsule.

The development and gradual specialization of the cells composing the vascular bundles and the various parts of the wall of the capsule, including the tubercle and pit, are indicated in the figures of capsules of different ages (figs. 19, 20, 21, 23, 28, 39). The development of the wall and bundles has been described for *P. globulifera* by MEUNIER (**16**), who failed, however, to note the basal tubercle or pit. One developmental feature that should be emphasized here is the curvature of the terminal portion of the peduncle. This is important because it determines the position of the mature capsule on the peduncle, which is different from that found in *P. globulifera* and from those found in most other Marsileaceae. In the two youngest sporocarps seen (figs. 14, 19), both of which already had well developed sporangia, the capsule portion stands nearly at right angles to the peduncle. It is believed that a very young sporocarp would, like that of *P. globulifera* (**11**, figs. 14, 31), show the peduncle and capsule lying in one straight line with the soral canals facing ventrally at right angles to this line. As development progresses, the dorsal side of the peduncle, near its upper end, continues to grow more rapidly than the ventral side (figs. 14, 16, 17), with the final result that the capsule is bent through 90° (figs. 17, 18) until it lies almost flat against the ventral side of its peduncle. Its closed soral canals thus come to point toward the stem at the base of the peduncle, instead of at right angles to this direction as they did earlier (figs. 18, 20, 22). This is the reverse of the position on its peduncle of the mature capsule in *P. globulifera*, in which the tips of the canals finally point very nearly upward (**11**, figs. 32, 33, 36). Another result of this curvature of the peduncle is that the morphological apex of the capsule, the location occupied by the apical cell when it was active, comes to lie on the side of the capsule away from its own peduncle (fig. 22; *cf.* **11**, figs. 14, 31, 33*A*). The different degrees of such curva-

ture of the peduncle found in other species of the Marsileaceae, with some of the misconceptions to which these have led, will be discussed in a later paper.

Although this bending is in the same direction as the circinate bending of the tip of the leaf, it is not really the equivalent of this, since the bending is confined to a single fixed zone of the peduncle, not to a constantly advancing zone. The terminal portion of this structure (the capsule itself), moreover, does not bend at all.

Development of the sporangia in *Pilularia minuta* could not be followed through from the beginning since the youngest sporocarps had sporangia with well formed, single-layered walls and with either spore mother cells or tetrads (figs. 15, 19, 20) already differentiated. These and the later stages seen offered no evidence of essential differences in the development of the sporangium and spore from those already described in detail (**16, 6**). All the sporangia initiated mature and produce functional spores. There is no indication of even the initiation of more than one megasporangium and two microsporangia per sorus, which might suggest the derivation of this sporocarp from one with a larger number of sporangia like that of *P. globulifera* or of *Marsilea*.

The structure and development of the capsule of *P. minuta*, as here described, indicate its close correspondence with that of the capsule of *P. globulifera*. Like the latter it is similar in all essential respects to the capsule of *Marsilea quadrifolia*. The adherence of this capsule of *P. minuta* to the *Marsilea* plan is shown by the equivalent dorsiventral (zygomorphic) symmetry; by the arrangement of the placentas and their vascular bundles; by the structure of the dorsal pit and tubercle; and by the overlapping of two layers of the thickened hypodermis inward from the pit. Finally the study of all the stages of development that were available showed these to correspond essentially to the homologous stages of the capsule of *P. globulifera* and so to those of *Marsilea*. This comparison of the capsule of *P. minuta* with those of other Marsileaceae shows it to be the simplest one of the family. This simplicity is indicated: by the reduction of the number of sori to two, the smallest number possible if the zygomorphic symmetry of the *Marsilea* type of sporocarp is to persist at all; by the reduction of the number of megasporangia in

each sorus to one and of microsporangia usually to but two; and by the correlated reduction of the number of branches of the vascular bundle in the capsule and the lack of anastomoses of the ventral tips of the branches such as occur regularly in *Marsilea* and in other species of *Pilularia*. It is thought that this simplicity is a secondary one that has been derived by a process of reduction from a type of vascular system like that of *Marsilea quadrifolia* or *M. brownii*, and that forms like *M. aegyptiaca* (3), *Regnellidium* (14), and *Pilularia globulifera* illustrate successive stages in this reduction process. There seems adequate reason also, from the structure of the leaf as well as of the sporocarp, for believing that *Marsilea* species with four pinnae and numerous sori, like *M. quadrifolia*, are the most primitive members of the family Marsileaceae (2).

## Summary

1. Although *Pilularia minuta* has been known for a century it is rarely collected and has been little studied, its development practically not at all.

2. The plant is small and has a delicate stem and a leaf but half a millimeter thick and a few centimeters long. Each node of the stem bears a leaf, a bud or branch, one or more roots, and, when fruiting, nearly every one also bears a single sporocarp. The vascular bundle of the latter is attached directly to that of the stem, close beside that of the leaf. The initial cell of the root and probably that of the stem also is tetrahedral, while the initial of the leaf is bifacial. The leaf shows no rudiment of a lamina at any stage of its development. Delicate trichomes, each with a 2-celled stalk and a tapering limb of 4–6 square-ended cells, cover all young organs except roots.

3. The sporocarp is rather long stalked, with a brownish, ovoid capsule, the tip of which, by a bend of the peduncle, is finally brought to face toward the base of the latter. The capsule has a basal pit and a tubercle just below it, at the very end of the peduncle; while on the opposite side of the capsule, at the ends of the soral canals, are two nipple-like papillae. There is but one sorus on each side (in each locule) of the bent capsule, each with one megasporangium toward the base and two microsporangia toward the tip. The single megaspore of each sorus is uninucleate when mature, and

is densely stored with large starch grains. The fibrovascular system of each of the two locules consists of three meridional branches that do not fuse at their ventral ends as in most other Marsileaceae. In fact the middle branch in each locule ends altogether at the placenta, half way to the tip of the capsule.

4. The firm wall of the capsule consists of a slightly thickened epidermis and the hypodermal and subhypodermal layers of much thickened prismatic cells. The hypodermis is doubled, by the overlapping of two narrow strips on the median line, just above the place where the single vascular bundle from the peduncle penetrates the base of the capsule. Both peduncle and capsule of the young sporocarp show that they have been developed by the activity of a bifacial initial, like that actually observed in the leaf of this species.

5. The indusium and the lateral walls of the capsule, which together surround the soral cavity and thus finally bury the sporangia deep in the capsule, are evidently formed, as in *Pilularia globulifera*, by the more rapid growth ventralward of all cells of the ventral side of the capsule except the sporogenous cells themselves.

6. The zygomorphic symmetry of the sporocarp is unmistakable. It is evident in such external features as the bending of the peduncle; the flattening of the capsule against its peduncle; the median position on its surface of the tubercle and the pit at its base; and the pair of soral papillae at the tip of the capsule. This symmetry is also emphasized by the whole internal structure of the capsule, for example: by the right and left sori with the indusium about each; by the forking of the single median vascular bundle just after entering the capsule; by the median position of the overlap of the hypodermis in the basal wall of the capsule; and finally this same bilateral symmetry is evident through the whole history of development as far back as this could be traced. It is because the likewise unmistakable bilateral symmetry of the capsule of *P. globulifera* has been almost universally overlooked that the symmetry of the present species has been so emphasized.

7. The plan and structure of the capsule of *Pilularia minuta*, when compared with that of *P. globulifera*, indicate close correspondence in nearly every point except those in which such correspondence is rendered impossible by the differences in the size of the capsules, in

the number of sori, and in the number of sporangia in each sorus. Thus the basal pit and the tubercle below this; the peculiarities of the epidermal, hypodermal, and subhypodermal layers; and finally the overlapping of the hypodermis at the base of the capsule of *P. minuta*, all closely resemble in character these same structures as they are developed (although generally overlooked) in *P. globulifera*. The branching of the vascular bundle in each locule of the capsule of *P. minuta* is on the general plan of that in *P. globulifera* but decidedly simpler. In many of these features also this capsule resembles that of *Marsilea quadrifolia*, although the similarity here is not so close as that between the two species of *Pilularia*.

8. In general conclusion, it is evident that *Pilularia minuta* is the simplest of all the Marsileaceae; and comparison with other species indicates that it is the most reduced member of this family. Its simplicity is indicated, in the first place, by the vegetative organs, including their size and external and internal differentiation. In the second place it is shown by such features of the structure of the sporocarp as (1) the presence of but two sori, the smallest number possible if the zygomorphic symmetry of the *Marsilea* type of sporocarp is to persist at all; (2) the reduction of the number of megasporangia in each sorus to one and of the number of microsporangia to two; and (3) the correlated reduction in the number of branches of the vascular bundle in the capsule and the lack of anastomoses of the ventral tips of certain of these branches (such as occur regularly in *Marsilea* and in all other known species of *Pilularia*). With all this simplification, its adherence to the general plan as in *Marsilea* is still shown by its clearly dorsiventral (or bilateral) symmetry; by the detailed structure of the basal pit and tooth; and by the overlapping of two layers of the thickened hypodermis beneath the basal pit. Finally, study of all the available stages of development showed these to correspond essentially to equivalent stages of the capsule of *P. globulifera*, and so, of course, to those of *Marsilea*.

9. The writer believes that this simplicity in structure of the sporocarp of *P. minuta* is a derived one, which has probably been reached by a reduction from the more primitive, although more complex, type found in the capsule of *Marsilea quadrifolia* and *M. brownii*. This reduction is evident in the small size of the capsule

and in the general plan and the internal differentiation of the capsule as a whole. Perhaps, however, it is most clearly indicated by a comparison of the vascular bundle systems of the capsules in different species of this family. The simplest bundle system in the whole family is that just described for *P. minuta*, in which each locule has two complete but not anastomosing meridional branches and one incomplete branch that ends in the placenta of the sorus. This is regarded as derived ultimately from the much more complex and more primitive one of the species of *Marsilea* referred to. The different types of bundle system found in the capsules of such species as *M. polycarpa* (4), *Regnellidium* (14), *M. aegyptiaca* (4), and *P. globulifera* (16) probably illustrate successive steps in this process of simplification or reduction of the bundle system, which ends, so far as is now known, with the bundle system of *P. minuta*.

10. In confirmation of the implications of this evidence from the fertile segment of the leaf, it may be recalled that the external differentiation of the foliage leaf in the Marsileaceae has been generally admitted to indicate definitely that *Marsilea*, whose petiole or rachis has four alternating pinnae at the end, is the most primitive (that is, most fernlike) genus of the family (2). *Regnellidium* with but two pinnae, and *Pilularia* with no pinnae at all, must then be regarded as exemplifying downward steps, or reductions, in the evolution of the leaf in this family. It is clear, therefore, that the common trend of these several different sorts of evidence points to *P. minuta* as the most reduced, on the whole, of all the known Marsileaceae.

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# FUSARIUM WILT AND CORM ROT OF FREESIAS[1]

J. J. TAUBENHAUS AND WALTER N. EZEKIEL

(WITH TWENTY-FIVE FIGURES)

## Introduction

The growing of freesias for cut flowers has developed into a highly specialized enterprise. The parent corms are planted in the open during the late winter or early spring months in many southern states. The new crop of corms, harvested in early summer, is then shipped back to eastern and western states and planted in greenhouses to produce flowers largely for the Christmas trade. According to MORGAN,[2] florists alone purchase from 20 to 25 million corms each year for forcing purposes. Further large numbers are sold for home and estate plantings. Frequently as many as 50 million freesia corms are sold in the United States during the year.

During 1924, some Indiana growers attempted for the first time to produce their supply of freesias from Texas-grown corms. Plantings were made for several years at Donna (Hidalgo County) in the Lower Rio Grande Valley. After encouraging results at first, the crops were attacked in 1926, and even more seriously in 1927, by a disease which caused heavy losses. In the study of this disease, the writers have been assisted by growers at Donna, Texas, who have furnished land and labor, and by florists in Indiana, California, and Florida, who have furnished corms for inoculation purposes.

There appears to be little or no previous information about this disease in the literature. HEALD[3] reported a bulb rot of common freesias which he considered due to a species of *Fusarium*. Diseased corms from Texas have been submitted to Dr. HEALD, and in

[1] Published with the approval of the Director as Contribution no. 239, Technical Series, of the Texas Agricultural Experiment Station.

[2] MORGAN, W. P., A note on the hybridization of freesias. Proc. Indiana Acad. Sci. 1930.

[3] HEALD, F. D., Bulb rot of common freesia. U.S. Dept. Agric. Bur. Plant Ind. Plant Disease Reporter (mimeographed). Suppl. 65. 421. 1928.

a letter of June 5, 1929, he stated that the freesia disease from Texas appeared different from that found in Washington.

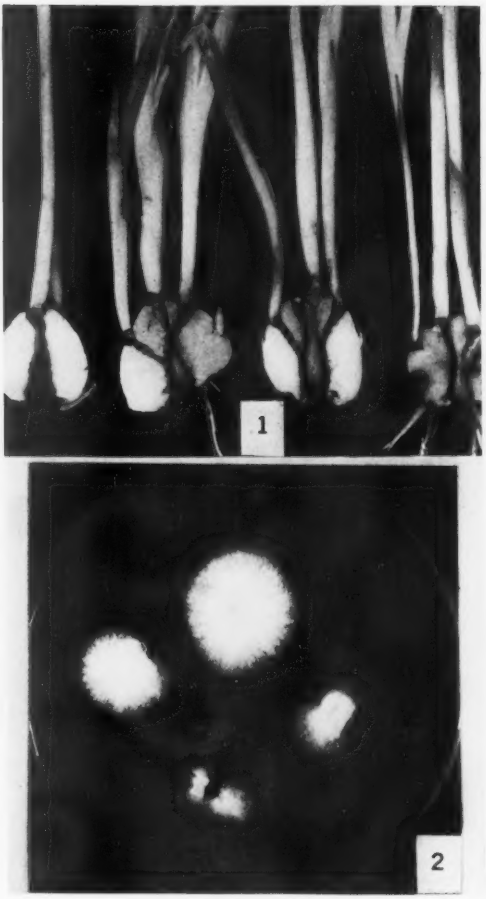
Information about freesia diseases in California was kindly furnished by Professor ELIZABETH SMITH[4] of the California Experiment Station: "Professor RALPH SMITH and I did quite a bit of work on freesias some years ago while the industry was active at Santa Cruz. . . . . The plants seemed to go down in a general rotting, usually starting at the crown and working up and down. . . . . This we finally decided was due to a species of *Fusarium*. . . . . The growers at Santa Cruz moved out to the Santa Barbara district shortly after, so that no more work has been done on the problem."

The occurrence of this disease in other states has been definitely established from diseased freesia corms received from California, Florida, and Indiana. It is probably present also in every other state where the plants are grown extensively for the market.

LOSSES.—Some evidence of the heavy losses caused by this wilt and corm rot was obtained in counts of infected plants and corms in the field, and in inspection of commercial corms in the laboratory at College Station. During 1927, 85 per cent of the plants in several plantings of freesias at Donna, Texas, were destroyed outright by the wilt, and the corms from the remaining apparently healthy plants were already partially decayed. Of some 2000 corms from an infested field at Donna, only 13 per cent were apparently normal, 72 per cent had definite but as yet small lesions, and 15 per cent showed advanced decay.

Of a shipment received from California on November 30, 1931, containing 450 corms, 18 per cent were apparently normal, 75 per cent showed definite early outside lesions, 5 per cent showed an advanced stage of core rot, and 1 per cent were completely decayed. Other shipments from California were badly affected with corm rot also. Corms received from Florida and from Indiana were in no better condition. A statement from a Florida grower is of interest: "In going over my freesias it is almost impossible to find those that have no indication of trouble. There is a discoloration on all of them. . . . . The freesias from the Pacific Coast are infected also."

[4] In a letter dated October 18, 1927.




FIGS. 1, 2.—Corm rot of freesias: fig. 1, longitudinal sections of sprouting corms showing infection spreading from center through vascular system and girdling base of new growth; fig. 2, *Fusarium* growing out from portions of infected corms, in petri-dish culture.

**Description of the disease**

The following description is based on observations of the disease under field conditions at Donna, Texas, and additional observations of diseased corms received from California, Florida, and Indiana.

In the field the disease usually appears in spots before the plants bloom and continues to spread until time of harvest. The scattered infested areas throughout the field coalesce eventually into large dead spots 40 to 50 feet or more in diameter. At first a few plants appear yellowish, then gradually wilt, die, and collapse on the ground (fig. 25). With collapse of the affected plants, the yellow color of the leaves disappears completely and the dead foliage takes on a straw to papery-white color. The dead leaves gradually decay as they lie on the ground and in time disappear. Plants adjoining infected ones on either side are still erect and normal, but in a few days they too gradually take on the characteristic yellow color, collapse, and die.

The disease attacks not only the tops, but also spreads into the main roots, the larger laterals, and the feeding rootlets, all of which take on a typical pink color. These roots finally decay and assume a dark brown color which in a short time becomes obscured by a superficial coating of the mycelium and spores of a *Fusarium* sp. So far as noticed, infected roots do not soften but remain firm and practically intact although discolored and dry. Infection apparently occurs near the crown of the plant. The top may die only after the disease has invaded the underground parts and then spread into the stem; or the top may die as an initial result of infection before the disease has extended into the corms.

From the infected and dead roots the disease spreads to the corms, which may be found in various stages of infection. With relatively mild infection, the husks may conceal the few or many superficial to deep pinkish lesions on the outside of the corms (figs. 3, 4, 5). These lesions may be the result either of infection from the outside, spreading into the corm, or of infection from the inside spreading outward. In the latter case the heart or interior of the corm is invariably discolored and decayed, although firm (fig. 6). Corms with definite internal decay may usually be recognized by the

FIGS. 3–8.—Corm rot of freesias: fig. 3, corms with husks removed to show external lesions; figs. 4–8, longitudinal sections of infected corms: 4, with external lesions; 5, with more advanced decay arising from external lesions; 6, with decay confined to core and starting from plate of corm; 7, various stages of corm rot; 8, normal corm at right, cracking characteristic of advanced decay at left, and intermediate stages between.

fact that the central root clings to the plate of the corm, while the roots pull out readily from a normal corm and leave a clean scar.

The interior of a normal corm is white and of a mealy appearance. In diseased corms, however, the tissue becomes first yellowish and then, as decay progresses, there appears a definite dark brown discoloration confined at first to the center of the corm. We have designated this type of injury as "core rot." From the center the disease spreads through the ramifications of the vascular system (figs. 1, 6). In well advanced stages the browned vascular system is usually surrounded by a pink discoloration. Occasionally some yellow color also is found in the more advanced decayed tissue in the heart of the corm. In advanced stages of core rot, the center of the corm often cracks and deep cavities form which become filled with an almost pure growth of a *Fusarium* sp. (figs. 7, 8). As decay spreads and involves the entire corm, the affected tissue becomes of uniform chalky-white color, and of a hard, dry, friable, gritty texture, crumbling readily under light pressure. The writers have used the term "chalky dry rot" for this type of infection. The chalky type of dry rot occurs not only as the final stage of decay of entire corms, but also in small pockets arising from initial external infections.

### Cause of the disease

From the beginning of these studies it appeared evident that the cause of the trouble was probably a *Fusarium* sp., since this organism was nearly always found on the surfaces of dead roots, on badly decayed corms, and in cracks in the decayed corms. Isolations were made from surface-sterilized blocks of infected tissue of freesia corms grown respectively in Texas, California, Florida, and Indiana (fig. 2). As indicated in table I, the organisms obtained almost without exception were colonies of *Fusarium* spp., with only occasional occurrence of *Penicillium*, *Rhizoctonia*, *Verticillium*, or *Trichoderma*.

Corms for inoculation purposes were received from Indiana, California, and Florida. They were carefully examined, and it was found necessary to cull out large numbers of diseased corms before enough passably normal ones could be obtained for inoculation purposes. Inoculations were made with four *Fusarium* species isolated repeatedly from the infected corms from Texas, California,

Florida, and Indiana, and identified by Dr. C. D. SHERBAKOFF as *F. solani*, *F. bulbigenum*, *F. moniliforme*, and *F. martii-minus* respec-

TABLE I

ISOLATION CULTURES FROM DIFFERENT STAGES OF FREESIA CORM ROT (VARIETIES PURITY AND FLORAL TREASURE)

| SOURCE OF MATERIAL AND DATE OF CULTURES | STAGE OF DECAY | NO. OF PIECES OF INOCULUM | RESULTS AFTER 7–14 DAYS | |
|---|---|---|---|---|
| | | | NO. OF FUSARIUM COLONIES | NO. OF COLONIES OF OTHER ORGANISMS* |
| Donna, Texas, May 1, 1929 | Early core rot | 95 | 73 | 3 R, 5 P |
| | Medium core rot | 77 | 64 | 12 P, 7 V |
| | Advanced chalk rot | 191 | 125 | 10 P, 1 T |
| Donna, Texas, May 7, 1929 | Superficial lesions | 125 | 92 | 0 |
| | Chalk pockets | 200 | 163 | 12 P |
| | Early core rot | 175 | 114 | 22 P |
| | Advanced core rot | 125 | 120 | 19 P |
| | Advanced chalk rot | 180 | 112 | 0 |
| Indianapolis, Indiana, Sept. 10, 1929 | Superficial lesions | 250 | 117 | 0 |
| | Early core rot | 100 | 81 | 11 P |
| | Advanced core rot | 125 | 107 | 9 P |
| | Advanced chalk rot | 105 | 84 | 14 P |
| Donna, Texas, June 3, 1930 | Superficial lesions | 100 | 96 | 5 R |
| | Early core rot | 100 | 105 | 9 P |
| | Advanced core rot | 90 | 78 | 7 P |
| | Advanced chalk rot | 70 | 63 | 12 P |
| Costa Mesa, California, April 14, 1930 | Superficial lesions | 175 | 99 | 3 P |
| | Chalk pockets | 175 | 133 | 0 |
| | Early core rot | 70 | 63 | 0 |
| | Advanced core rot | 95 | 87 | 3 P |
| | Advanced chalk rot | 60 | 52 | 0 |
| Carlsbad, California, Nov. 5, 1930 | Superficial lesions | 200 | 173 | 0 |
| | Early core rot | 200 | 187 | 0 |
| | Advanced core rot | 100 | 98 | 0 |
| | Advanced chalk rot | 100 | 100 | 0 |
| Fern Park, Florida, Oct. 28, 1930 | Superficial lesions | 140 | 125 | 0 |
| | Chalk pockets | 126 | 106 | 14 P |
| | Early core rot | 100 | 97 | 3 P |
| | Advanced core rot | 75 | 62 | 1 P |
| | Advanced chalk rot | 105 | 69 | 4 P |

* P, *Penicillium;* R, *Rhizoctonia;* T, *Trichoderma;* V, *Verticillium.*

tively. Additional species tested were *F. batatatis* from sweet potatoes, *F. lycopersici* from tomatoes, *F. conglutinans* from cabbage, *F.*

*niveum* from watermelons, *Fusarium* sp. from Panama wilt of bananas (all furnished by Dr. SHERBAKOFF); and *F. vasinfectum* from cotton, and a *Fusarium* sp. from decayed gladiolus corms.

All corms used in these tests were first disinfected for 5 minutes in 1–2000 bichloride of mercury in 25 per cent alcohol, then rinsed in sterilized water and thereafter handled aseptically. Inoculations were made by three different methods. In one series, pure cultures of the organisms, grown on whole freesia corms in flasks, were mixed with soil previously steam-sterilized. Four 5-inch pots were used for each culture, and five normal corms were then planted in each pot of inoculated soil. In another series, some selected corms were inoculated with bits of pure culture inserted in punctures made with a sterile needle; and other corms were inoculated by dipping them into suspensions of spores from the pure cultures. The inoculated corms were incubated in large dry chambers at room temperature. Non-inoculated checks were used for each series.

It is to be noted from table II that all the *Fusarium* species isolated from freesias, and in addition *F. conglutinans* and *F. lycopersici*, caused typical damping-off of the plants as well as completely destroying the new roots and causing typical corm rot (figs. 15–23). *F. vasinfectum* caused typical corm rot but did not kill the plants. The other three species of *Fusarium* tested, *F. batatatis*, *F. niveum*, and the species from gladiolus, produced only a decay of the plate of the corm and of the main rootlets, without destroying the top of the plant. In direct puncture inoculations of the corms in moist chambers, however, it was found (table III) that all the species tested could cause typical core rot. Some superficial lesions were observed even on the check, uninoculated corms, but this was not surprising since the corms used in the experiments were merely the less obviously diseased ones selected from lots in which considerable natural infection was present. Considering the very much larger percentages of infection secured with all the *Fusarium* inoculations, however, there can be little doubt but that these fungi were actually responsible for the typical damping-off and corm rot symptoms produced on the inoculated plants. The organisms used in inoculation were finally recovered from the artificially inoculated plants (figs. 9–14).

It appears of special interest that a number of species of *Fusarium*,

in addition to the four isolated originally from freesia corms, have proved definitely pathogenic to freesias. Freesia corms apparently offer an exceptionally favorable medium for species of *Fusarium*,

TABLE II

RESULTS OF PLANTING FREESIAS IN STERILIZED SOIL INOCULATED WITH VARIOUS FUSARIUM SPECIES. SOIL INOCULATED DEC. 17, 1930; CORMS PLANTED JAN. 15, 1931; FINAL NOTES TAKEN FEB. 20, 1931

| INOCULUM | PERCENTAGE INFECTION | EFFECTS OF DISEASE | | |
|---|---|---|---|---|
| | | MOTHER CORMS | NEW ROOTS | TOP GROWTH |
| Fusaria isolated originally from diseased freesia corms: | | | | |
| F. bulbigenum from corms from | | | | |
| Donna, Texas | 100 | Typical core rot | Complete decay | Dwarfing, then damping-off |
| Carlsbad, Calif. | 100 | " " " | " " | " " " " |
| Fern Park, Fla. | 100 | " " " | " " | " " " " |
| F. martii-minus from corms from | | | | |
| Indianapolis, Ind. | 100 | " " " | " " | " " " " |
| Donna, Texas | 100 | " " " | " " | " " " " |
| Costa Mesa, Calif. | 100 | " " " | " " | " " " " |
| Fern Park, Fla. | 100 | " " " | " " | " " " " |
| F. moniliforme from corms from | | | | |
| Donna, Texas | 100 | " " " | " " | " " " " |
| Fern Park, Fla. | 100 | " " " | " " | " " " " |
| F. solani from corms from | | | | |
| Costa Mesa, Calif. | 100 | " " " | " " | " " " " |
| Indianapolis, Ind. | 100 | " " " | " " | " " " " |
| Donna, Texas | 100 | " " " | " " | " " " " |
| F. batatatis from decayed sweet potatoes | 100 | Decay of plate of corms only | " " | Dwarfing, trace of damping-off |
| F. conglutinans from wilt-infected cabbage plants | 100 | Typical core rot | " " | Dwarfing and damping-off |
| F. lycopersici from wilt-infected tomato plants | 100 | " " " | " " | " " " " |
| F. niveum from wilt-infected watermelon plants | 100 | Decay of plate of corms only | " " | Dwarfing only |
| F. sp. from decayed gladiolus corms from Calif. | 100 | Decay of plate of corms only | " " | Dwarfing, trace of damping-off |
| F. vasinfectum from infected cotton plants from Brazos Co., Texas | 100 | Typical core rot | " " | Dwarfing, trace of damping-off |
| None, check | 0 | Normal | Normal | Normal |
| " " | 0 | " | " | " |

and this is no doubt the cause of the widespread occurrence of the wilt and corm rot. It may be anticipated that further study will demonstrate that many more species of *Fusarium* are capable of causing decay of freesias.

It is not uncommon to find the corms, in various stages of natural decay, covered with various other fungi, notably *Trichoderma*, *Penicillium*, and *Rhizoctonia*. These three organisms were inoculated on

TABLE III

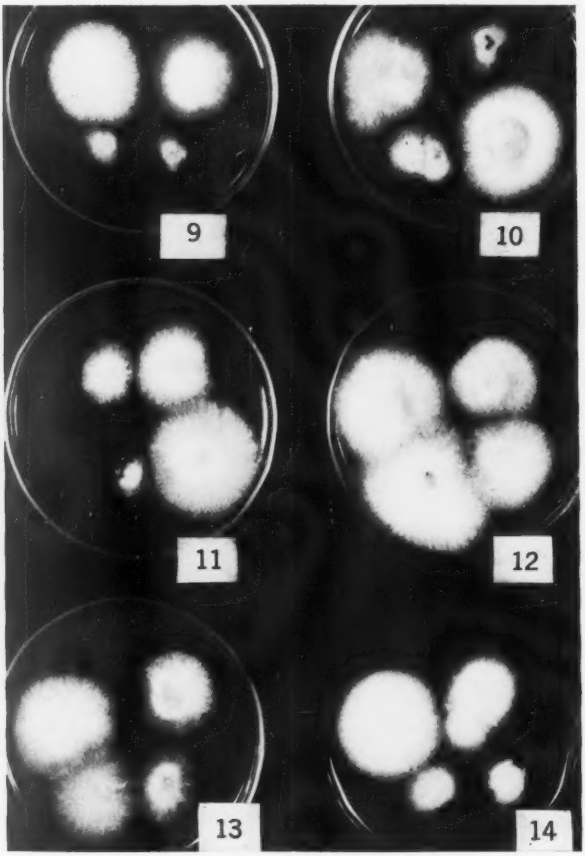
INOCULATION OF INDIVIDUAL FREESIA CORMS (VARIETY SPLENDENS) WITH FUSARIUM SPP., 75 CORMS PER SERIES, IN LARGE DRY PETRI DISHES KEPT AT ROOM TEMPERATURE; NOVEMBER 20–DECEMBER 23, 1930

| INOCULUM | METHOD OF INOCULATION | RESULTS AFTER ONE MONTH | |
|---|---|---|---|
| | | PERCENTAGE CORMS INFECTED | REMARKS |
| Fusaria isolated originally from diseased freesia corms: | | | |
| F. bulbigenum from corms from | | | |
| Donna, Texas | N† | 100 | Typical core rot |
| Fern Park, Fla. | N | 100 | " " " |
| " " " | S | 100 | *Superficial lesions |
| Carlsbad, Calif. | S | 100 | " " |
| F. martii-minus from corms from | | | |
| Donna, Texas | N | 100 | Typical core rot |
| Carlsbad, Calif. | N | 100 | " " " |
| " " | S | 100 | Superficial lesions |
| Fern Park, Fla. | S | 100 | " " |
| F. moniliforme from corms from | | | |
| Donna, Texas | N | 100 | Typical core rot |
| Fern Park, Fla. | N | 100 | " " " |
| " " " | S | 100 | Superficial lesions |
| Carlsbad, Calif. | S | 100 | " " |
| F. solani from corms from | | | |
| Donna, Texas | N | 100 | Typical core rot |
| Costa Mesa, Calif. | N | 100 | " " " |
| " " " | S | 100 | Superficial lesions |
| F. batatatis from sweet potato plants | N | 58 | Typical core rot |
| | S | 60 | Superficial lesions |
| F. lycopersici from tomato plants | N | 75 | Typical core rot |
| | S | 40 | Superficial lesions |
| F. niveum from watermelon plants | N | 50 | Typical core rot |
| | S | 60 | Superficial lesions |
| F. sp. from Panama wilt of bananas | N | 90 | Typical core rot |
| | S | 82 | Superficial lesions |
| F. vasinfectum from cotton plants | N | 86 | Typical core rot |
| | S | 80 | Superficial lesions |
| None, check | N | 0 | Normal |
| | N | Trace | Superficial lesions |

* Superficial lesions resulting from surface inoculations were typical but less than 2 mm. in diameter.
† N, inoculation through needle punctures; S, inoculation by dipping corms in suspension of spores.

normal corms, but the moist decay produced was unlike that caused by the *Fusarium* spp.

It seemed of interest to determine whether the *Fusarium* species which cause freesia core rot might also be able to infect other bulbs.




FIGS. 9–14.—Reisolation of *Fusarium* spp. from inoculated freesia plants shown in figs. 16–21.

One inoculation experiment was run with Bermuda onions, which were washed in tap water, dipped for 10 minutes in 1–2000 $HgCl_2$ in 25 per cent alcohol, and then rinsed twice in sterilized water. The

inoculum was placed in slits in the two outer scales, in three different places on each bulb. As shown in table IV, of the four species tested, only *Fusarium solani* and *F. martii-minus* were able to cause decay of inoculated onion bulbs. The symptoms observed were totally unlike those caused on inoculated freesia corms. *F. solani* and *F. martii-minus* were recovered from the respective inoculated onion bulbs.

TABLE IV

INOCULATION OF ONION BULBS WITH FUSARIUM SPP. ISOLATED ORIGINALLY FROM DISEASED FREESIA CORMS; 15 BULBS PER SERIES. INOCULATED FEB. 26, 1931; FINAL NOTES TAKEN MARCH 26, 1931; BULBS KEPT IN DRY CLOSED CHAMBERS

| INOCULUM | PERCENTAGE INFECTION | EFFECT ON ONION BULBS |
|---|---|---|
| F. bulbigenum from corms from Donna, Texas | 0 | All bulbs sound |
| F. martii-minus from corms from Carlsbad, Calif. | 100 | Moist but solid decay, involving $\frac{1}{3}$ of bulb |
| F. moniliforme from corms from Carlsbad, Calif. | 0 | All bulbs sound |
| F. solani from corms from Donna, Texas | 100 | Moist but solid decay, involving $\frac{1}{3}$ of bulb |
| Check, uninoculated | 0 | All bulbs sound |

In another experiment, normal gladiolus corms were planted in soils previously heavily inoculated respectively with *F. solani*, *F. bulbigenum*, *F. martii-minus*, and *F. moniliforme*, all isolated from infected freesia corms. With all four species of *Fusarium*, the plates of the corms were attacked by a decay which in no case penetrated deeply into the interior of the corms. This localized infection, however, prevented development of new roots from the diseased plate. It would therefore appear unsafe to plant gladiolus in soil infested with the *Fusarium* species which cause freesia corm rot.

### Soil, soil screenings, and freesia corms as disease carriers

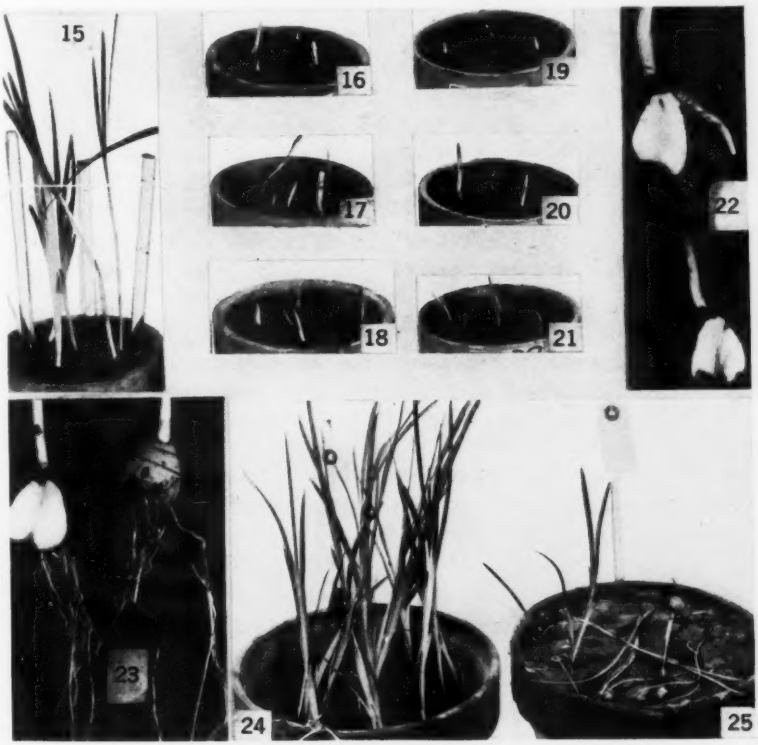
A quantity of infested soil was secured from an infested field at Donna, Texas. The soil, a typical Victoria clay loam, was dug out to a depth of 8 inches and shipped in sacks to College Station in August, 1930. Upon arrival, some 48 hours after shipment, it was carefully screened through a quarter-inch sieve to remove pieces of infected freesia roots and corms. The screenings were saved for an-

other experiment to be mentioned presently. The sifted soil was then placed in 50 5-inch pots, of which 25 were steam-sterilized for 4 hours at 10 pounds' pressure and the remainder left unsterilized. On September 5, 1930, both series of pots were planted with disinfected, apparently normal corms, about ten corms to each pot. Growth started apparently normally in both series; but after 5 weeks the plants in the unsterilized soil began to show typical symptoms of yellowing and damping-off, while none of the plants in the steam-sterilized soil appeared to be attacked (figs. 24, 25). After 3 months the plants were removed from the soil for final examination. Those from the steam-sterilized soil showed only occasional slight infection on the roots and on the exterior of the parent corms themselves. This infection was presumably carried with the parent corms. On the other hand, 80 per cent of the plants from the unsterilized soil had succumbed to typical damping-off, and typical core rot was found on the new corms which were produced in these pots.

It was of interest to determine further whether a soil, virgin as to freesia culture, would also carry the infection. Accordingly, Lufkin fine sandy loam material was secured from one of our cotton plats at College Station. The soil was carefully sifted to remove roots and other trash, and the sifted soil material was placed in 24 5-inch pots, of which half were steam-sterilized. Both series of pots were then planted with normal freesia corms. Not a single case of corm rot or wilt appeared on the plants which grew in the sterilized soil, while definite lesions and discolorations were found on the new corms in the unsterilized soil. However, the typical yellowing and damping-off was absent. This mild infection was apparently caused by organisms occurring in the virgin soil.

In California and other freesia-growing areas, the growers have noticed that continued growing of freesias in infested soil results in heavier losses from the disease. A California grower, for instance, writes that freesias planted on new land where nothing had been grown for at least seven years suffered only 5–10 per cent loss, while at least 75 per cent of the crop died out on old land which had been planted with freesias for the two preceding years. The small percentage of disease on the new land was probably due to introduction of the disease with the corms which were used for planting or to the various other *Fusarium* species already present in the soil.

It has been stated previously that screenings of infected and dead freesia roots and corms were removed from the soil secured from Donna. These screenings were mixed with steam-sterilized soil,




FIGS. 15–25.—Inoculation experiments with freesias: fig. 15, normal check plant; figs. 16–21, dwarfing resulting from inoculation with various *Fusarium* spp.; fig. 22, longitudinal section of typical dwarfed plants showing early stage of corm decay and destruction of all roots; fig. 23, longitudinal section and exterior view of normal corms and roots from check plants shown in fig. 15; fig. 24, normal plants growing in steam-sterilized soil from infested freesia field; fig. 25, damping-off type of injury resulting from planting of normal corms in soil from same source.

placed in ten 5-inch pots, and planted with disinfected, apparently sound, freesia corms. As checks, apparently normal corms were planted in ten 5-inch pots of sterilized soil. At the end of the experi-

ment, 90 per cent of the plants in the pots with the screenings from the infested freesia soil had succumbed to typical damping-off and core rot. In the check pots, only a trace of the plants showed exterior symptoms of core rot lesions.

Fifty freesia corms with superficial lesions were selected at random from the crop of an infested field at Donna, Texas. The husks were removed and the corms washed in tap water, disinfected for 5 minutes in 1–2000 $HgCl_2$ in 25 per cent alcohol, and rinsed twice in sterilized water. These disinfected corms were then planted on September 8, 1930, ten corms to each of five 8-inch pots of steam-sterilized soil. All of the corms germinated apparently normally; but after 18 days, the plants in all the pots ceased growing and gradually lost the normal green color. In 12 more days, the plants in all five pots had wilted and died. The symptoms were similar to those observed on plants in the field. A check planting of 50 apparently normal corms from Indiana remained healthy with the exception of two plants.

## Summary

1. *Fusarium* wilt and corm rot of freesias is prevalent in California, Florida, and Indiana, and has become destructive in Texas with the introduction of extensive freesia growing. Four species of *Fusarium*, *F. bulbigenum*, *F. martii-minus*, *F. moniliforme*, and *F. solani*, were isolated from infected freesia corms, and reproduced the disease in inoculation experiments. *F. conglutinans* from cabbage plants and *F. lycopersici* from tomato plants also induced typical symptoms on inoculated freesias; and five other *Fusarium* species were found able to cause somewhat less severe injury. It is probable that other species of *Fusarium* may at times cause wilt and corm rot of freesias. The disease is carried by infected corms, by old remnants of infected plants, and by the soil.

2. *Fusarium solani* and *F. martii-minus* caused decay of inoculated onion bulbs, but the other two *Fusarium* spp. isolated from freesia corms did not attack onions. Normal gladiolus corms planted in soil inoculated with the four species of *Fusarium* from freesias were attacked by a decay of the plates, which also prevented development of new roots.

AGRICULTURAL EXPERIMENT STATION
COLLEGE STATION, TEXAS

# MACROSPOROGENESIS AND EMBRYOLOGY OF MELILOTUS[1]

D. C. COOPER

(WITH PLATES II, III)

## Introduction

Perhaps the earliest detailed study of the embryo sac of *Melilotus* is that by GUIGNARD (**7, 8**), who described the embryo sacs of a number of the Leguminosae. YOUNG (**16**) figured a series of stages in the development of the embryo sac and embryo of *M. alba*. In the short paper accompanying the plates he reported that "in *Melilotus* the macrospore mother cell develops into the embryo sac without first undergoing tetrad division." CASTETTER (**2**) gave a detailed account of microsporogenesis and of the development of the pollen grain. He was especially interested in the "furrowing" process in the formation of the microspores. SMITH (**12**) confirmed the chromosome count as given by CASTETTER. ELDERS (**5**), FRYER (**6**), and CLARKE (**3**) have counted the chromosomes in the root tips of a number of species of *Melilotus* and have found the same diploid number, 16, in all the species examined.

## Materials and methods

Material from two species of sweet clover, *Melilotus alba* and *M. officinalis*, and from the variety Redfield Yellow (which is possibly a derivative of a hybrid between those two species),[2] was used in this investigation. Buds, open flowers, and young fruits were taken from plants growing in the Genetics greenhouses at the University of Wisconsin during the late winter and early spring of 1932. These were fixed in La Cour's chromo-acetic-osmic fixative, in Licent's solu-

[1] Papers from the Department of Botany and the Department of Genetics (no. 150), Agricultural Experiment Station, University of Wisconsin. Published with the approval of the Director of the Station.

[2] Unpublished results of investigations by R. A. BRINK. Acknowledgment is due Mr. L. W. KEPHART, Division of Forage Crops and Diseases, U.S. Department of Agriculture, Washington, D.C., for furnishing the Redfield Yellow plants used from the Department stock at Redfield, S.D.

tion (80 cc. 2 % chromic acid, 5 cc. glacial acetic acid, 15 cc. commercial formalin), in Flemming's medium solution, and in Karpechenko's modification of Nawaschin's fluid. Since the young buds tend to float on the surface of an aqueous solution, they were first dropped into Carnoy's alcohol-acetic-chloroform fluid for a short time (10–20 seconds) and then transferred to the fixative, in which they immediately sank. The older flowers were dissected, and the pistils removed and fixed separately. For stages in fertilization, pistils were fixed at periods 18, 21, 27, 32, 36, 40, and 45 hours respectively after pollination. Karpechenko's modification of Nawaschin's fluid and Licent's solution gave the best results with the embryo sacs and with stages in embryo development. In the latter fluid the endosperm was particularly well fixed.

After imbedding in paraffin, sections were cut 8–12 $\mu$ in thickness, mounted serially, and stained in Haidenhain's iron-alum haematoxylin, Ehrlich's haematoxylin, or a combination of the latter with light green.

No significant differences were found between the species studied so far as concerns the chromosome number or the history of the macrospore mother cell, the macrospores, and the embryo sac. Since the material for the study of fertilization and embryo development was limited to the variety Redfield Yellow, the description which follows is based chiefly upon the work with that variety.

## Investigation

DEVELOPMENT OF OVULE.—The ovules arise in two rows, one on each side of the ventral suture of the ovary. There are usually two ovules in each row, which are spaced far enough apart so that they alternate with those of the adjoining row. As many as six ovules have been found in an ovary, but the usual number is four. The young ovule is initiated by the multiplication of the hypodermal cells of the placenta. Protuberances are thus pushed out into the cavity of the ovary, and, as these continue to develop, cell division becomes more active on one side than on the other, with the result that the ovule bends toward the base of the ovary. REEVES (**10**) found that in *Medicago* the direction of this curvature may be determined mechanically by the growth of the carpel. The young ovule

is orthotropous, according to him, until it comes in contact with the dorsal wall of the ovary; then it begins to bend, usually toward the base. In *Melilotus* the curvature of the ovule begins before its nucellus touches the dorsal wall of the ovary, and no part of the ovule is in contact with the dorsal wall until it is almost mature.

The ovules usually curve toward the base of the ovary, although in a few instances it has been noted that the apical ovule curved toward the stylar end of the ovary. This is similar to the condition in *Medicago* (**10**) and very different from that in *Phaseolus* (**15**), where all the ovules curve toward the apex of the ovary.

The inner integument arises from the epidermis of the ovule at a level just basal to the macrospore mother cell, at about the time that that cell is in an early heterotypic prophase stage (fig. 4). Shortly thereafter the outer integument arises from the epidermis at a level just below the inner integument (figs. 5, 6). Both integuments, each composed of two layers of cells, develop so as nearly to cover the nucellus. The outer integument grows more rapidly than the inner one and reaches a level almost even with the apex of the nucellus by the time the four macrospores are formed (fig. 11). The outer integument continues to grow more rapidly on that side of the ovule away from the placenta, and the curvature of the ovule continues so that the micropyle lies parallel to the funiculus while the ovule is still at right angles to the latter and the inner integument has just reached the level of the apex of the nucellus (fig. 15). The ovule is typically campylotropous by the time the embryo sac is mature (fig. 17). At that stage the outer integument is massive in structure in the region of the micropyle, whereas the inner integument is only two cell layers in thickness and has grown over the apical end of the embryo sac so as to leave a very short inner portion of the micropyle.

At an early stage in the development of the ovule, and at about the time that it begins to curve, a hypodermal cell at the apex of the nucellus becomes differentiated as an archesporial cell (fig. 1). This cell divides to form an inner sporogenous and an outer parietal cell (figs. 2, 3). The outer parietal cell divides further, forming additional nucellar cells. It is usually divided longitudinally only, a single layer of parietal cells thus being produced (figs. 4, 5). Occasionally it divides once transversely (fig. 7) and then longitudinally, so that two

layers of parietal cells are formed. The sporogenous cells of *Melilotus* are not so deeply imbedded in the nucellus as is the case in other Leguminosae studied by GUIGNARD (**7, 8**), MARTIN (**9**), and REEVES (**10**).

The primary sporogenous cell is easily recognized by its greater size (fig. 3), by the greater size of its nucleus, and by its peculiar staining properties. Only one sporogenous cell appears in the nucellus of the Redfield Yellow variety and in *M. officinalis*, although in most instances two or three such cells are found in *M. alba*. These multiple sporogenous cells have much the same appearance as those described by REEVES for *Medicago sativa*.

MACROSPOROGENESIS.—The primary sporogenous cell (fig. 18) becomes the macrospore mother cell without further division. It increases in length as well as in diameter, so that at its greatest size it is approximately twice as long as wide. In the early heterotypic prophase the nucleus of the macrospore mother cell contains one conspicuous nucleolus imbedded in the chromatic network. This network condenses toward one side of the nucleus to form a dense synizetic knot (fig. 19). On recovery from synizesis, loops of paired threads spread out toward the nuclear membrane (fig. 20) until the open spireme stage is reached. Transverse segmentations of the open spireme form the chromosomes, which early appear as pairs of threads (fig. 21). These shorten and thicken until a typical diakinesis stage is reached wherein 8 pairs of chromosomes can be counted (figs. 22, 24).

The chromosomes of *Melilotus*, as seen on the equatorial plate of the heterotypic division, are very similar in size and shape. Eight pairs can be distinguished in both metaphases and anaphases (figs. 25, 26).

After the heterotypic division, the macrospore mother cell is divided by means of a cell plate in such a manner that the basal cell is somewhat larger than the apical one. This cell division is not directly transverse but is at an oblique angle to the longitudinal axis of the cell (fig. 9). After the homoeotypic division a row of four macrospores is formed. The plane of the second cell division is at an oblique angle to that of the first, so that the macrospores may not appear to be in a linear row (figs. 10, 27). The formation of four macro-

spores was also observed in *Melilotus alba* (fig. 12) and *M. officinalis* (fig. 13). GUIGNARD (**7, 8**) observed the formation of only three macrospores in *Phaseolus multiflorus* and *Medicago arborea*. WEINSTEIN (**15**) likewise found that only three macrospores are formed in *Phaseolus vulgaris*. Most observers of the Leguminosae, however, have found a row of four macrospores. There is some variation as to the position of the functional macrospore. GUIGNARD found that the third macrospore from the micropylar end of the tetrad functions in several species of *Acacia*, and SAXTON (**11**) found a similar situation in *Cassia tomentosa*. In *Melilotus*, as in the majority of the Leguminosae thus far examined, however, it is the chalazal macrospore that functions in the formation of the embryo sac.

CHROMOSOME NUMBER.—CASTETTER (**2**) reported the haploid number of chromosomes of *Melilotus alba* as 8. This count was verified by SMITH (**12**). ELDERS (**5**) counted the chromosomes in the root tip cells of three species, *M. alba*, *M. alba annua*, and *M. officinalis*, and found the diploid number to be 16. FRYER (**6**) reported the diploid number as 16 in *M. sulcatus* and *M. indica*, as well as in those species already studied. He found that one pair of the chromosome complement of *M. alba* is distinguished by the presence of satellites. CLARKE (**3**) also found 16 to be the diploid number of chromosomes in *M. wolgica*, *M. italica*, *M. segetalis*, and *M. messanensis*.

The haploid number of chromosomes is 8 in Redfield Yellow as well as in *M. alba* and *M. officinalis*. These counts were made in the microspore mother cells and in the macrospore mother cells of Redfield Yellow. At early diakinesis a pair of satellite chromosomes is present in this variety (fig. 22). In late diakinesis, and on the heterotypic equatorial plate, the chromosomes are more or less rounded and the satellites are not distinguishable (figs. 23, 25). A polar view of an equatorial plate stage of a nucleus of the endosperm shows 24 chromosomes. Satellites have been seen attached to two of these chromosomes (fig. 41).

DEVELOPMENT OF EMBRYO SAC.—The chalazal macrospore functions as the embryo sac mother cell, and the other three macrospores disintegrate (fig. 27). By three nuclear divisions an 8-nucleate cell is formed (figs. 14, 15, 28). Cell walls cut off three small cells, each

with a single nucleus, at either end of the embryo sac, leaving one large middle cell with a nucleus near each end, the central region being highly vacuolate (fig. 16). During this time the parietal cells surrounding the embryo sac are disintegrating, so that it ultimately comes to lie in contact with the epidermal cells of the nucellus.

These observations are at variance with those of YOUNG (**16**), who reported the "lily" type of macrogametophyte development for *M. alba*. In preparations of both *M. alba* and *M. officinalis*, as well as in Redfield Yellow, a row of four macrospores was characteristic (figs. 11, 13). The embryo sac in each case develops from the chalazal macrospore and the other macrospores disintegrate. A comparison of figure 14, showing a 2-nucleate macrogametophyte, with YOUNG'S figure 15 would indicate that they are at approximately the same stage of development. In both figures disintegrating nucellar cells are present, whereas at the time of the heterotypic and homoeotypic divisions (figs. 7, 10) the nucellar cells have not yet begun to break down.

After the formation of the 7-celled embryo sac, a considerable time elapses during which the ovule develops rapidly and the embryo sac elongates until it is several times as long as wide (fig. 17). In this process the epidermal cells of the nucellus in the micropylar region break apart and the embryo sac grows out so that ultimately more than a third of it is in direct contact with the cells of the inner integument. WEINSTEIN found, in *Phaseolus*, that some of the nucellar cells form a cap or protecting layer over the apical portion of the embryo sac. Such a layer is not present in *Melilotus*.

The cells of the nucellus at the chalazal end of the embryo sac are broken down and digested in the process of elongation, so that the basal portion of the embryo sac becomes deeply imbedded. The continued curvature of the ovule causes the embryo sac to be bent at an angle in its middle region. The antipodals usually disintegrate before fertilization occurs. GUIGNARD (**7**) found that the antipodals of the same species of *Melilotus* may disintegrate early in some embryo sacs and persist until after fertilization in others. The basal polar nucleus moves toward the micropylar end in the denser cytoplasm along one side of the large central vacuole, and comes to lie in close contact with the apical polar nucleus in a region close to the egg apparatus

(fig. 29). The polar nuclei fuse to form the primary endosperm nucleus before fertilization (fig. 30).

The egg apparatus consists of three elongated pear-shaped cells, the egg being somewhat larger than the two synergids. In each synergid a large vacuole appears in the basal region, and the nucleus is imbedded in the dense cytoplasm just above this vacuole and in the middle region of the cell. Just before fertilization, the micropylar ends of the two synergids elongate considerably and extend into the micropyle. The cytoplasm in this region contains minute elongated vacuoles or canals which extend forward from the region of the large basal vacuole (figs. 30, 32). These vacuoles or canals together with dense areas in the cytoplasm give the appearance of a filiform apparatus. This is similar to the condition described by BROWN (1) for *Phaseolus vulgaris* and by the writer (4) for *Lycopersicon esculentum*. A transverse section of the egg apparatus at a level containing the synergid nuclei shows the vacuoles and denser regions in the cytoplasm (fig. 31). There are no dense accumulations in contact with the cell walls and the walls themselves show no peculiar markings.

The broad end of the egg extends beyond the synergids into the embryo sac. There is a large vacuole in the pointed apical region of the egg, and the large egg nucleus is buried in the denser cytoplasm at the base of the cell.

Fertilization takes place between 18 and 21 hours after pollination. In this process a single pollen tube grows down the micropyle and enters the embryo sac between the synergids and the egg. The synergids are not destroyed by the entrance of the pollen tube and may persist until an embryo of some size is formed. WEINSTEIN found that the antipodals and synergids of *Phaseolus vulgaris* disintegrate early, and that at the time of fertilization the embryo sac consists of but two cells, the egg and the primary endosperm cell. This early disintegration of antipodals and synergids has been reported for a number of the Papilionoideae. In *Melilotus*, as already stated, the antipodals usually disintegrate before fertilization, but the synergids persist for some time thereafter. The two male nuclei are discharged from the pollen tube near the egg. Figure 32 shows the micropylar end of an embryo sac at the time of fertilization. One male gamete nucleus is in close proximity to that of the egg and the

other male nucleus is closely appressed to the fused polar nuclei. The male gamete nuclei are not spherical as in *Phaseolus* (**15**), but are somewhat elongated and a nucleolus is clearly evident near one end of each. Figures 33 and 34 show the process of fusion of a male gamete nucleus with the egg nucleus. Two nucleoli are clearly evident in both figures.

The primary endosperm nucleus divides shortly after fertilization, and a number of nuclei are to be found in the cytoplasm of the embryo sac at the time of the division of the zygote nucleus (fig. 35). YOUNG, MARTIN, and WEINSTEIN report a similar situation in other Leguminosae. On the other hand, STRASBURGER (**14**) found that the zygote and primary endosperm nuclei divide simultaneously in *Lupinus*. Such a condition was also reported by GUIGNARD for *Phaseolus multiflorus*.

EMBRYO.—The zygote divides transversely (fig. 35) to form a 2-celled proembryo consisting of an enlarged basal cell and a smaller apical cell (fig. 36). The latter cell divides (fig. 37) to form a linear row of three cells (fig. 38). According to SOUÈGES (**13**), the apical cell of this row by further divisions forms the embryo in *Medicago* and the middle and basal cells divide to form the suspensor. In *Melilotus* a vertical division of the apical cell does not occur until a 4-celled proembryo has been formed (fig. 39). It may be, however, that the two middle cells of this proembryo arose by a division of the middle cell rather than of the apical cell present at the 3-celled stage. The embryo is formed from the apical cell of the 4-celled proembryo only, whereas the multicellular suspensor is formed by vertical and horizontal divisions of the stalk cells of the proembryo (figs. 40, 42). The suspensor persists even after disintegration of the endosperm, and fragments of it can be seen attached to an almost mature embryo (fig. 53).

Figure 39 shows the nuclear division in the apical cell of a 4-celled proembryo. Two further divisions occur, the first vertical (figs. 41*a*, 43*b*) and the second transverse, giving rise to an 8-celled embryo. Periclinal divisions now take place which cut off the dermatogen (figs. 44–47). Multiplication of the cells of the embryo continues, forming at first a spherical mass of cells (figs. 48, 49) which later becomes somewhat pear-shaped (fig. 50), the apical region being broader than the base.

The cotyledons first appear as two outgrowths of the apex of the embryo (figs. 51, 52). These appear simultaneously and are directly opposite each other. They elongate rapidly, forming two elongated, broad, and somewhat flattened structures. These cotyledons at maturity are very broad, flat, leaflike structures that are packed with storage materials.

The basal region likewise develops both laterally and terminally to form the hypocotyl. At the same time a conspicuous knob, the epicotyl, is formed at the apex of the embryo and between the bases of the cotyledons. The embryo curves during the course of its development so that ultimately the cotyledons and hypocotyl come to lie parallel to one another, the most acute part of the curvature being in the apical portion of the hypocotyl in the region of the epicotyl (fig. 53).

ENDOSPERM.—The nuclei of the endosperm are scattered throughout the embryo sac, but the greater number of them are in the region immediately next the embryo. Cell division begins first in that region of the endosperm surrounding the suspensor at about the time of the 16-celled stage of the embryo, and from this time on during the development of the embryo, cell walls are being formed in the region of the embryo and there are free nuclei in the chalazal end of the endosperm (fig. 51). The cell structure of the endosperm is somewhat spongy in character. In ovules containing almost mature embryos no trace of endosperm could be found.

## Summary

1. In *Melilotus* a single apical hypodermal cell is differentiated in the young ovule as an archesporial cell. This cell divides to form a primary parietal and a primary sporogenous cell.

2. The primary sporogenous cell functions as the macrospore mother cell in *M. officinalis* and in the Redfield Yellow variety of sweet clover. A number of sporogenous cells (1–3) are usually formed in *M. alba*, but only one of them undergoes further development.

3. In consequence of two divisions, the macrospore mother cell produces a row of four macrospores, the chalazal one of which develops into a 7-celled embryo sac of the usual type; the other three macrospores disintegrate.

4. The micropylar end of the nucellus breaks down and the em-

bryo sac elongates greatly, the basal portion becoming deeply imbedded in the nucellus and the apical portion extending so as to lie in direct contact with the inner integument.

5. The polar nuclei fuse and the fusion nucleus comes to lie just basal to the egg apparatus.

6. The antipodal cells disintegrate just prior to fertilization.

7. The apices of the synergids elongate and a distinct filiform apparatus is produced.

8. The pollen tube enters the embryo sac between the synergids and the egg, and the synergids are rarely broken down in consequence of fertilization but persist for some time thereafter.

9. Fertilization occurs under greenhouse conditions between 18 and 21 hours after pollination.

10. The zygote, by transverse divisions, forms a filament of three or four cells. The terminal cell of this filament forms the embryo. The basal cells by further divisions form a multicellular suspensor.

11. The primary endosperm nucleus divides before the zygote, and there are usually four or more free nuclei in the embryo sac at the time of division of the zygote.

12. Cell division in the endosperm begins in the region of the suspensor at about the time the embryo has reached the 16-celled stage.

13. The endosperm is entirely absorbed by the time the embryo is mature.

14. Remnants of the suspensor are still present after the absorption of the endosperm.

15. The haploid number of chromosomes is 8. *Melilotus alba* and Redfield Yellow each have one pair of satellite chromosomes.

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## EXPLANATION OF PLATES II, III

All drawings were made with an Abbé camera lucida at table level. Spencer compensating oculars, a Spencer 8 mm. N.A. 0.60 apochromatic objective, and a Leitz oil-immersion N.A. 1.32 objective were used.

### PLATE II

FIG. 1.—Young nucellus showing hypodermal archesporial cell. ×310.

FIG. 2.—Nucellus; archesporial cell divided to form primary sporogenous and primary parietal cell. ×310.

FIG. 3.—Young macrospore mother cell; early prophase. ×310.

FIG. 4.—Ovule showing origin of inner integument. ×310.

FIG. 5.—Ovule showing origin of inner and outer integuments. ×310.

FIG. 6.—Ovule; macrospore mother cell at diakinesis. ×310.

FIG. 7.—Ovule; macrospore mother cell at equatorial-plate stage, heterotypic division. ×310.

FIG. 8.—Somewhat later than fig. 9; heterotypic anaphase. ×310.

FIG. 9.—Daughter cells formed after heterotypic division. ×310.

FIG. 10.—Daughter cells formed after homoeotypic division. ×310.

FIG. 11.—Ovule with linear row of 4 macrospores. ×310.

FIG. 12.—Same stage as fig. 11; from *M. alba*. ×310.

FIG. 13.—Same stage as fig. 11; from *M. officinalis*. ×310.

FIG. 14.—Nucellus with 2-nucleate macrogametophyte; disintegrating macrospores and nucellar cells. ×310.

FIG. 15.—Four-nucleate macrogametophyte. ×310.

FIG. 16.—Nucellus with young 7-celled embryo sac. ×310.

FIG. 17.—Ovule at later stage; 7-celled embryo sac. ×145.

FIG. 18.—Macrospore mother cell; early prophase. ×1060.

FIG. 19.—Synizesis. ×1060.

FIG. 20.—Approaching open spireme stage. ×1060.

FIG. 21.—Nucleus showing chromosome pairs. ×1060.

FIG. 22.—Early diakinesis. ×1060.

FIGS. 23, 24.—Stages in diakinesis. ×1060.

FIG. 25.—Macrospore mother cell with heterotypic equatorial plate. ×1060.

FIG. 26.—Macrospore mother cell; heterotypic anaphase. ×1060.

FIG. 27.—Typical tetrad of macrospores, the three outer spores beginning to disintegrate. ×1060.

FIG. 28.—Embryo sac; divisions leading to 8-nucleate stage. ×625.

FIG. 29.—Apical portion of embryo sac showing micropylar apparatus and fusing polar nuclei. ×310.

FIG. 30.—Same as fig. 29, slightly later stage; polar nuclei fused and synergids beginning to elongate. ×310.

PLATE III

FIG. 31.—Transverse section of synergids and egg in region of synergid nuclei. ×625.

FIG. 32.—Stage in fertilization; one male nucleus closely appressed to primary endosperm nucleus and another in close proximity to that of egg. ×310.

FIG. 33.—Egg nucleus of fig. 32 in detail showing male nucleus closely adjacent. ×1060.

FIG. 34.—Stage in process of fusion of egg nucleus with male nucleus. ×1060.

FIG. 35.—Binucleate proembryo; cell in process of division. ×625.

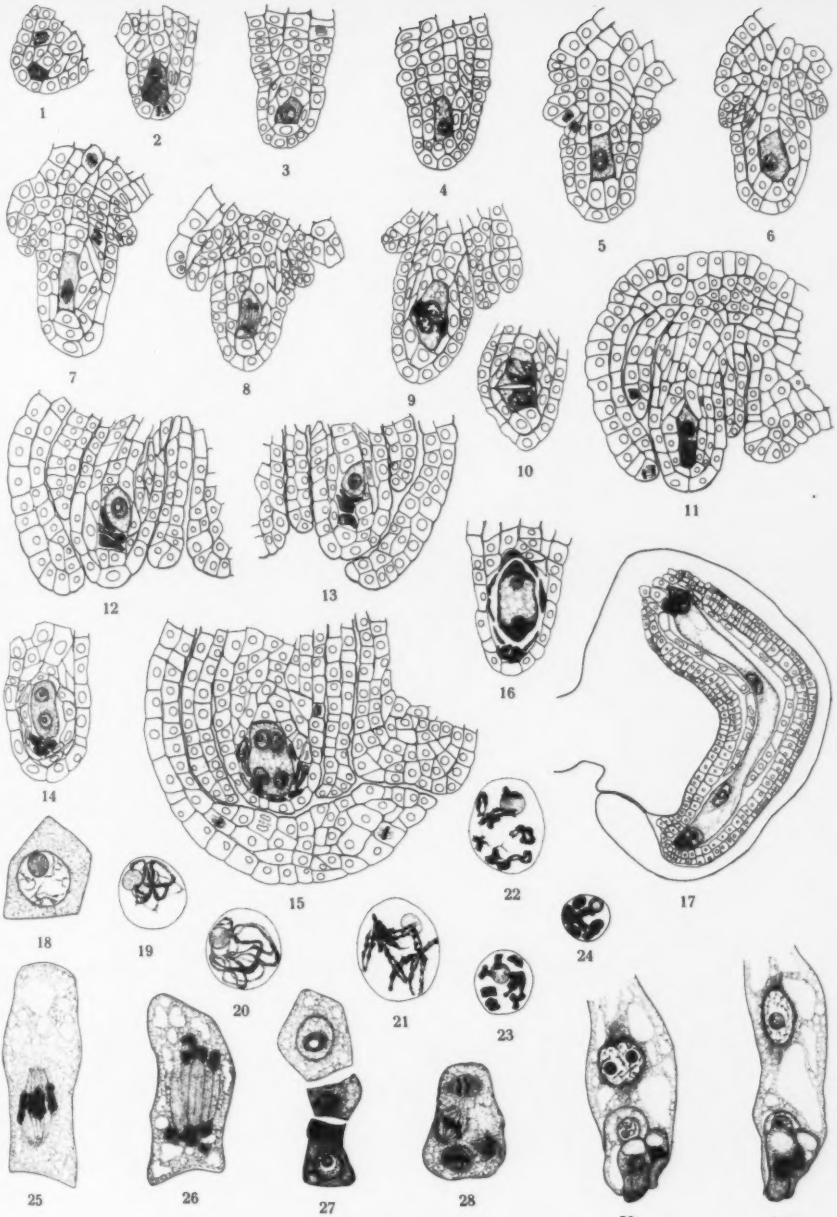
FIG. 36.—Two-celled proembryo; synergids still present and conspicuous. ×310.

FIG. 37.—Two-celled proembryo; nucleus of apical cell in process of division. ×310.

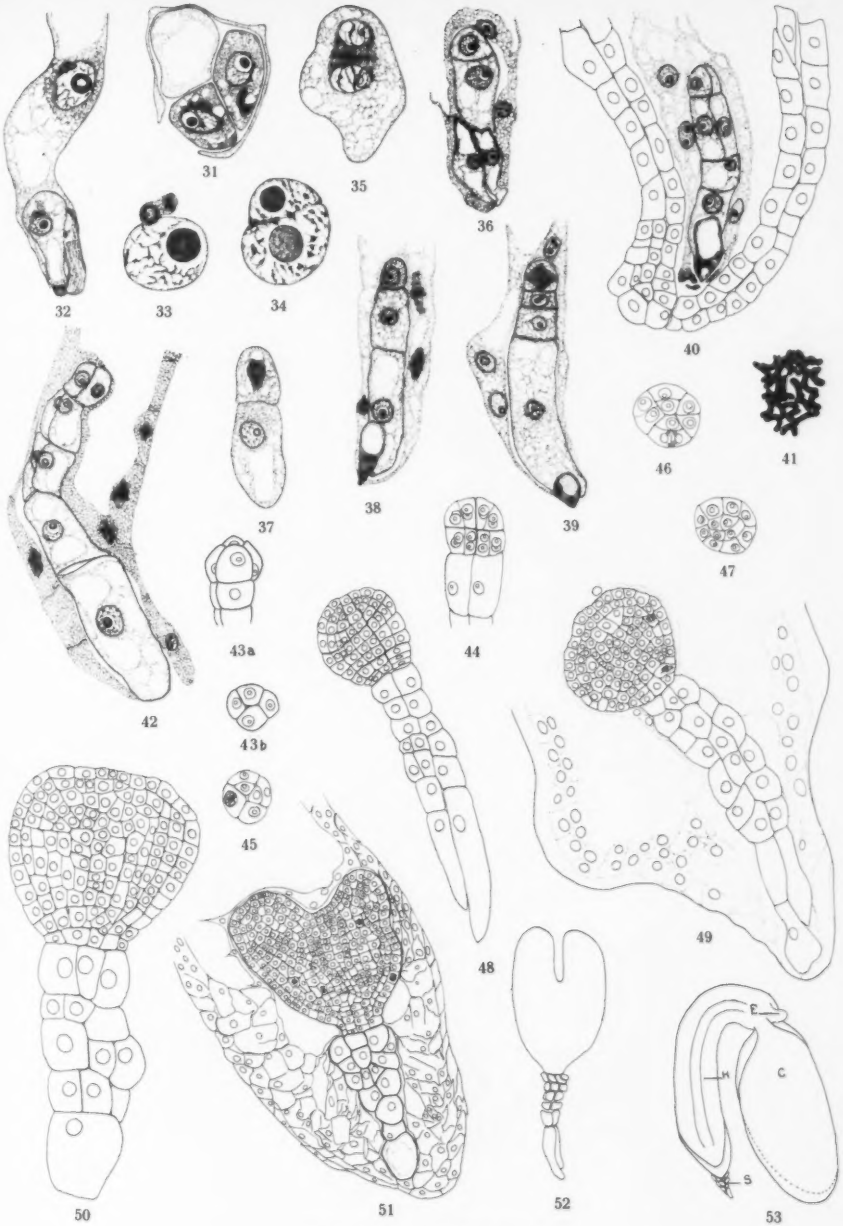
FIG. 38.—Three-celled proembryo; nuclear divisions in endosperm. ×310.

FIG. 39.—Four-celled proembryo; nucleus of apical cell dividing. ×310.

FIG. 40.—Somewhat later stage showing longitudinal division of apical cell and of adjacent suspensor cell. ×310.




COOPER on MELILOTUS




COOPER on MELILOTUS

FIG. 41.—Polar view of equatorial plate in endosperm; 24 chromosomes can be counted. ×1060.

FIG. 42.—Still later stage; further division of suspensor cells; nuclear divisions in endosperm. ×310.

FIG. 43.—Four-celled embryo: *a*, lateral view; *b*, polar view. ×310.

FIG. 44.—Young embryo with 16 cells. ×310.

FIGS. 45–47.—Transverse sections of upper quadrant of embryo showing periclinal divisions. ×310.

FIG. 48.—Older embryo showing well-defined regions and suspensor made up of large cells. ×220.

FIG. 49.—Older embryo, still spherical in shape and with cellular endosperm in the closely adjacent region. ×220.

FIG. 50.—Still older embryo, somewhat flattened apically. ×290.

FIG. 51.—Embryo showing early development of cotyledons; cells formed in the endosperm at apex of embryo; cells of endosperm in region of suspensor loosely arranged. ×145.

FIG. 52.—Embryo showing further development of cotyledons. ×70.

FIG. 53.—Almost mature embryo; remnants of suspensor still present: *c* cotyledons; *e*, epicotyl; *h*, hypocotyl; *s*, suspensor. ×20.

# DEVELOPMENT OF THE MALE GAMETOPHYTE IN TRADESCANTIA

KARL SAX AND H. W. EDMONDS

(WITH PLATE IV)

A study of the development of the male gametophyte in *Tradescantia* seems to show that nuclear differentiation is associated with a definite polarity of the microspore, and that in turn this polarity is correlated with the nuclear-cytoplasmic relations at the end of the meiotic divisions. The male gametophyte is favorable for such studies because of the comparatively simple structure, the striking difference in appearance and function of the two nuclei in the mature pollen grain, and the ease with which consecutive stages in development may be obtained.

This investigation was based on aceto-carmine smears of microspore mother cells and microspores at various stages of development. After mounting, the preparation was heated until a few small air bubbles appeared. This treatment results in a better differentiation between nucleus and cytoplasm. After the slide had been heated, it was inverted on a piece of filter paper and slightly pressed to remove the excess fixing fluid, and then sealed with a mixture of gum mastic and paraffin. Such preparations remain in good condition for several weeks, or even months, if kept in a refrigerator. Aceto-carmine smears were also prepared without heating, for examination of the cell granules which are destroyed by heat. Living microspores were also studied as a check on the fixed material. Some material was fixed and sectioned for an examination of cross-sections of microspores.

## Investigation

At the first meiotic division the chromosomes pass to opposite sides of the microspore mother cell, and the daughter nuclei are formed near the outer cell wall. Figure 1 is a photograph of a dyad showing the characteristic position of the nuclei. Both of the second meiotic division spindles are usually in the same plane and at right

angles to the plane of the first meiotic spindle. Again the daughter chromosomes pass as far as possible to opposite poles, so that the four nuclei are near the periphery of the tetrad. The nuclei at this stage are shown in figure 2. At the end of the second meiotic division, cell walls are found at right angles to the first, so that the cells of the tetrad are oriented like segments of an orange.

As the tetrad nuclei pass into the resting stage, they take a position near the center of each cell. At a later stage the one-nucleate microspores separate and become independent cells, although they tend to retain the characteristic shape found at the tetrad stage. The one-nucleate microspores, as seen from the side, are ovoid in shape, with one side flattened, this side being undoubtedly the central axis or inner wall of the cells at the tetrad stage. In smear preparations the microspores seldom lie on end so that their morphology in cross-section can be determined, but in sectioned material a cross-section of the microspore across the shorter axis shows an angular outline on one side. As the microspore usually lies on one of the two flattened walls, the flattened side is the angular junction of these two walls, while the outer or rounded side of the microspore is derived from the outer wall of the original microspore mother cell.

In these young microspores a number of refractive granules are especially conpicuous in the cytoplasm. These granules are found at prophase stages of meiosis and persist during the meiotic divisions. In the young microspores they seem to be scattered more or less at random in some cells, although as a rule they are found most abundantly on either side of the nucleus toward the ends of the cell. The granules are shown in figure 3, which is a photograph of a microspore which was not subjected to heat, which would have resulted in disappearance of the granules. As the nucleus enters the prophase stage the granules disappear, and the regions previously occupied by them appear as vacuolate areas.

The nature of these granules has not been determined. They do not seem to be starch grains because they are invisible under a polarizing microscope with crossed Nicolls, and they do not show the typical color reaction with a solution of iodine and potassium iodide. The solidity of the granules is demonstrated by the fact that they are not deformed by pressure on the cover glass, either before

or after extrusion from the cell. On collision they do not coalesce as would fluid droplets.

As the cytoplasmic granules disappear the microspore increases in size, and the flattened side of the cell becomes more conspicuous. It has a smooth wall as contrasted with the corrugated wall of the outer or curved side, and appears to be thicker (figs. 4–6). The nucleus, which at this time is in an early prophase stage, lies nearer the inner wall, at first nearer one end of the cell (fig. 5) but later taking a more central position (figs. 4, 6). During these prophase stages large vacuoles appear in the cytoplasm. In living cells they appear to be spherical, but fixation apparently destroys their smooth, regular outlines. The vacuole or vacuoles may extend from one side of the microspore to the other (fig. 4), but at later stages they are found usually at either side of the nucleus (figs. 6, 8, 9) and separated by the cytoplasm between the nucleus and the outer wall of the cell.

The chromosomes lie flat on the cell plate at the metaphase stage of the microspore division. Three of the chromosomes have approximately median fiber constrictions, while the other three have submedian constrictions (fig. 7). The dividing chromosomes are invariably nearer the inner or flattened wall of the microspore, and the axis of the poles is at right angles to this wall. The vacuoles are conspicuous on both sides of the dividing chromosomes (figs. 8–10).

As the chromosomes pass to the poles, those near the inner or flattened wall of the microspore remain compact, while those near the outer wall may elongate even before a nuclear wall is formed. The compact nucleus is cut off by a thin cell wall which includes very little cytoplasm. The other nucleus increases greatly in size and passes into the resting stage. This is the vegetative or tube nucleus (figs. 9–11). The vegetative nucleus seems to begin disintegration even before the pollen tube is formed, and apparently plays little part in further gametophytic development.

The small or generative nucleus never passes into a typical resting stage. As it enlarges it retains its staining reaction. The thin cell wall surrounding this nucleus is apparently broken as the nucleus rapidly elongates to form a slender sickle-shaped body. The generative nucleus at this stage no longer retains its position near the inner wall of the microspore, but may lie in any region of the cell.

The pollen tube germinates at one end of the microspore. The generative nucleus passes into the pollen tube, where it divides to form the two male gametes. Fertilization occurs about twelve hours after pollination.

### Duration of microsporogenesis and gametophyte development

The length of time for development of the different stages in microsporogenesis and gametophytic development has been determined in *Tradescantia reflexa*. The data were obtained by marking

TABLE I

Meiosis and microspore development in Tradescantia

| Day | Microscopic events | Macroscopic events |
|---|---|---|
| 1–4 | P.M.C. prophase | Anthers white |
| 5–6 | Meiosis | Anthers pale yellow |
| 7–8 | Growth of microspore | Anthers yellow |
| 9–10 | Vacuolation of microspore | ..... |
| 11 | Microspore division | Color appears on petals |
| 12 | Differentiation of nuclei | ..... |
| 13 | Elongation of generative nucleus | ..... |
| 14 | Mature pollen | ..... |
| 15 | Pollination, A.M.<br>Fertilization, P.M. | Flower opens, A.M.<br>Flower closes, P.M. |

the buds with India ink and making smears each day from other buds of the same size from the same plant, the work being done in the field. Considerable variation may be caused by weather conditions. The data are summarized in table I.

In the case of *Tradescantia* plants growing in the field during the summer months, there is evidence of little diurnal periodicity except in opening and closing of the petals. Although division occurs in both the microspores and in the microspore mother cells at all hours of the day or night, it was generally easier to obtain meiotic divisions between 8 and 11 A.M. and microspore divisions between 3 and 6 P.M. The species investigated are self-sterile, and fertilization occurs only when a flower is pollinated with compatible pollen.

### Microspore development in partially sterile plants

Several plants of *Tradescantia* have been found which have partially sterile pollen. In one plant (*T. edwardsiana*) the pollen steril-

ity is about 50 per cent, and is caused by non-disjunction of segmental interchange chromosomes (3). More than 80 per cent pollen sterility is found in the related genus *Rhoeo*. In both cases tetrad formation is apparently normal. No differences can be observed in the young one-nucleate microspores, all having the typical granular bodies. At a slightly later stage, however, two classes of microspores are evident. The microspores which develop into normal pollen grains lose their granules as they increase in size, and microspore division occurs. The microspores which do not develop retain the granular bodies and do not increase in size. The nucleus degenerates, and no division occurs. Sterility counts made as soon as these two classes of microspores could be distinguished are essentially the same as those made when the fertile pollen grains are mature. It is evident that chromosome deficiency is associated with failure of microspore development at a very definite growth stage.

## Discussion

In the formation of the tetrad in *Tradescantia*, the second divisions are at right angles to the first division, and the division in the microspore is at right angles to the preceding second division. The axis of the microspore division is across the shorter diameter of the cell, in the great majority of cases. At an earlier stage before microspore division, the nucleus lies at one end of the cell surrounded by cytoplasm, while the other end of the cell is vacuolate, with only a peripheral layer of cytoplasm along the cell wall. Before the division occurs the cell becomes bilaterally symmetrical, however, with the nucleus nearer the inner or flattened wall of the microspore. Apparently a definite polarity exists which determines the axis and location of the spindle, although the cytoplasm and nucleus do not become oriented until shortly before division occurs. The polarity of the microspore is presumably determined at the preceding meiotic divisions.

The microspore division occurs in such a way that one cell is formed near the inner wall of the microspore and is cut off with little cytoplasm by a thin wall. This cell does not pass into a typical resting condition but elongates and becomes the generative nucleus. The other nucleus, surrounded by the bulk of the cytoplasm of the

cell, passes into the resting stage and apparently plays no further active part in microspore development. It would appear that the nuclear-cytoplasmic relations might be responsible for the differential development of the two nuclei, but until experimental modification of these relations can be made no definite conclusions are justified. It is clear, however, that nuclear differentiation, in respect to both structure and function, is associated with the position of the nuclei in the microspore.

Development of microspores in partially sterile plants of *Tradescantia* is of interest in respect to the relations between the chromosome complement and development. In this genus a complete haploid set of chromosomes is essential for normal gametophytic development, as is generally the case in plant species. Gametophytes with a haploid complement plus additional chromosomes seem to develop normally, and mature pollen grains are formed. For instance, in a triploid *Tradescantia* nine chromosomes were usually found at the microspore division (1). It is known that one-nucleate microspores with less than six chromosomes are sometimes formed, but none has been found with less than the haploid set at the time of microspore division in *Tradescantia*.

Plants containing segmental interchange chromosomes are usually partially sterile, resulting from non-disjunction, so that each daughter nucleus at the first meiotic division is deficient for a section of a chromosome and duplicate for another segment, although six chromosomes are present. These deficient nuclei in *Tradescantia* and *Rhoeo* are able to divide at the second meiotic division, and one-nucleate microspores are formed, but there is no further development. The nucleus remains in the resting stage and the cytoplasmic granules do not disappear as they do in normal microspores. It is evident that the absence of a chromosome or section of a chromosome prevents normal development of the cell. But why, if a deficient nucleus is able to divide once, does it fail at the time of the next division?

If the chromosome releases some gene product that passes from the nucleus to the cytoplasm with disintegration of the nuclear wall, the cytoplasm at the beginning of the first meiotic division will receive a complete set of genic material. The deficient nuclei formed

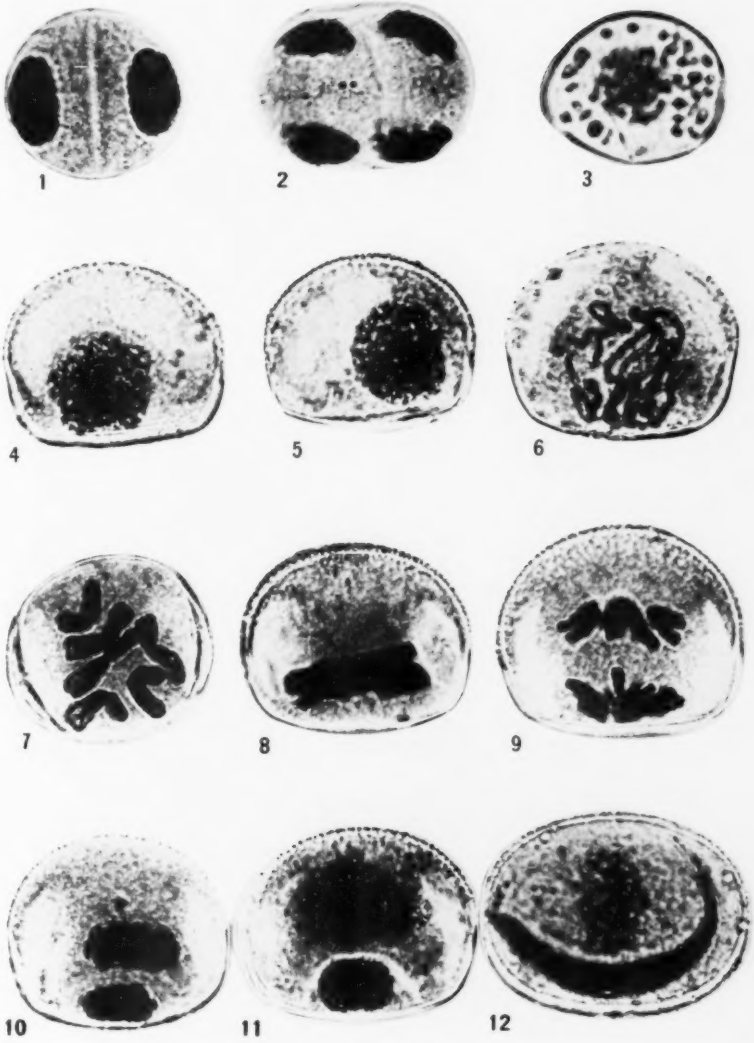
at telophase will be surrounded by "normal" cytoplasm, and this cytoplasm would in no way inhibit the second meiotic division. But the genic material released at the prophase of the second meiotic division would be deficient, so that the microspores would contain "deficient" cytoplasm. All the four microspores resulting from non-disjunction at the first meiotic division would then be deficient for certain essential gene products, and no further development could occur. The theory is plausible, of course, although it is possible that other factors are involved.

Evidence from experimental embryology seems to indicate that there is often a time element required for the reaction between the genes and the cytoplasm (**2**). This time lag may not be the same in different organisms nor in different stages of development. As the chromosomes become more differentiated, it is probable that each chromosome, or even a small segment of a chromosome, is more essential for the normal development of the individual. The cytoplasmic mass and amount of genic material released at each division may also be factors which determine the time when the cytoplasm responds to gene action.

## Summary

1. Development of the male gametophyte in *Tradescantia* seems to be correlated with a polarity established during the meiotic divisions. The spindle of the first microspore division is almost invariably oriented across the short axis of the cell, and the dividing nucleus is nearer the cell wall formed during meiosis. The nucleus formed near this inner cell wall becomes the elongated generative nucleus which divides in the pollen tube to form the two male gametes. The other cell, surrounded by most of the cytoplasm in the microspore, becomes the vegetative or tube nucleus and remains in the resting condition. Development is associated with a movement of the microspore nucleus before division and the formation of characteristic vacuoles.

2. Non-disjunction in segmental interchange species results in chromosome deficiencies in the dyad nuclei, but these nuclei divide normally at the second division, even though they are deficient for a chromosome segment. But the deficient one-nucleate microspores do not develop further. It is suggested that development of the




SAX & EDMONDS on TRADESCANTIA

microspore is dependent on a full complement of gene products which pass into the cytoplasm at the preceding division.

3. A brief chronology of meiosis and microspore development is presented. About two weeks elapse between early meiotic prophase and the formation of mature pollen grains in *Tradescantia reflexa*.

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## EXPLANATION OF PLATE IV

FIGS. 1–12.—Photographs of microspores of *Tradescantia gigantea* from aceto-carmine preparations. ×950.

FIG. 1.—Dyad showing position of daughter nuclei at end of first meiotic division.

FIG. 2.—Tetrad stage.

FIG. 3.—One-nucleate microspore showing granules in cytoplasm. Sterile microspores seldom develop beyond this stage.

FIGS. 4, 5.—Microspore showing relations of nuclei and vacuoles.

FIG. 6.—Prophase of microspore division. Nucleus always lies near flattened wall of microspore at this stage.

FIGS. 7, 8.—First microspore divisions.

FIG. 9.—Late anaphase of microspore division showing characteristic position of nuclei.

FIGS. 10, 11.—Differentiation of nuclei. Small nucleus near inner wall invariably produces the generative nucleus. Vegetative or tube nucleus enlarges and passes into resting stage.

FIG. 12.—Mature pollen grain showing elongated generative nucleus and disintegrating vegetative nucleus.

# NEW CASES OF APOGAMY IN CERTAIN HOMOSPOROUS LEPTOSPORANGIATE FERNS

W. N. Steil

(WITH SIX FIGURES)

## Apogamy in Pteris flabellata Thunb.

The first case of apogamy in a *Pteris* species was reported by Farlow (2) in *Pteris cretica* var. *albo-lineata*. The writer (6) reported apogamy in ten *Pteris cretica* varieties and in *P. sulcata* L., *P. quadriaurita* Retz. var. *argyrea* Moore, and *P. parkerii*, in which apogamy was described the following year. This paper now reports the discovery of apogamy in *P. flabellata* Thunb.

DeBary (1) investigated the spores of a number of *Pteris* species, including those of *P. flabellata*, and reported the sporophyte to be of non-apogamous origin.

The cultural conditions under which most of the prothallia for this investigation were grown, not only of the *Pteris* species but also of the other ferns in which apogamy is reported, have been described elsewhere (7). In some instances the prothallia were grown on the surface of a sterilized nutrient solution in small Erlenmeyer flasks. Since the embryo is always produced apogamously under ordinary cultural conditions, there can be no doubt in regard to the origin of the embryo of *Pteris flabellata*.

The prothallia of this species become typically heart-shaped (fig. 1) and in most respects resemble those of other species of *Pteris* in which apogamy has so far been described. Antheridia are usually produced in large numbers on the smaller prothallia, and occasionally on the larger ones, including those bearing embryos of apogamous origin. The antherozoids are actively motile and apparently normal. Archegonia have not been observed on any of the many prothallia examined.

When the embryo is about to make its appearance, it begins its development on the ventral side of the prothallium and a short distance posterior to the apical notch (fig. 2). Just previous to the first visible evidence of the formation of the embryo, however, an-

other group of small cells appears in the apical notch of the prothallium (fig. 3). During the early stages of development of the embryo, the small group of cells in the apical notch usually produces a projection (figs. 1, 4, 5). This projection frequently develops a tongue-like or cylindrical process, which, when the prothallia are grown in subdued light, may extend forward for a considerable distance. In some instances apogamous embryos were observed to develop on this outgrowth. The embryos thus produced were not always complete, forming a well developed leaf but often lacking a definite root.

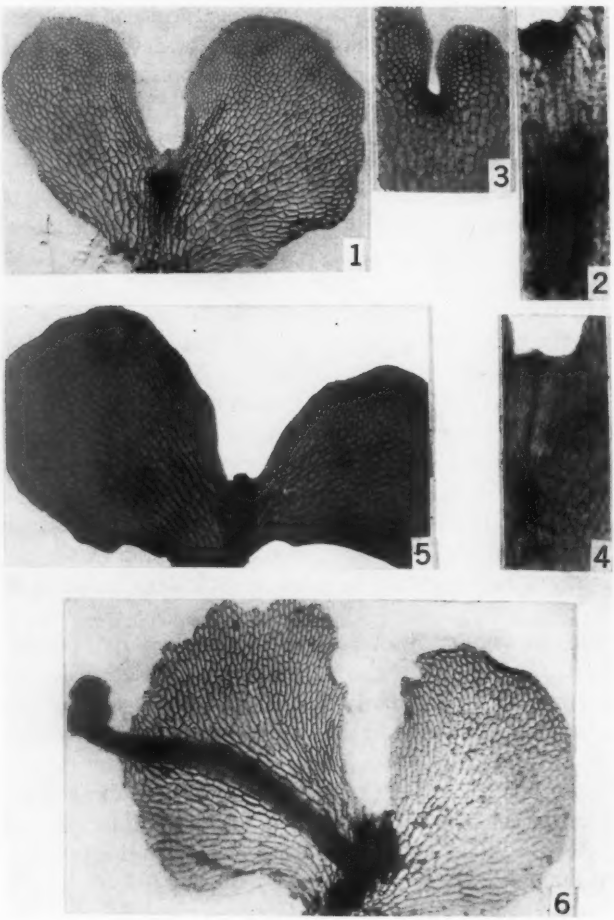
At an early stage in the development of the embryo, tracheids appear in the prothallium, usually a short distance posterior to the apical notch. These vascular elements can be distinguished at first as single isolated cells (fig. 2), but frequently as a compact group of several cells. The tracheids are produced in a considerable portion of the prothallium. Sometimes they extend forward to the apical notch (fig. 4). In a few instances two strands of tracheids were observed to extend toward the apical region of the gametophyte. The presence of these sporophytic tissue elements in a fern prothallium was first reported by LESZCYC-SUMINSKI (1848) in *Pteris sulcata*.

In the order of development of parts of the embryo, the leaf appears first (fig. 5), then the root, and finally the stem. Often a leaf was observed to develop far in advance of the root (fig. 6). No unusual features were found in the later stages of development of the embryo.

The prothallium increases considerably in size during the early stages of development of the embryo, and it continues to enlarge even after the leaf of the embryo has grown upward above the surface of the prothallium.

### Apogamy in Pellaea cordata (Cav.) J. Sm.

In 1905 GOEBEL found apogamy in *Pellaea nivea*. Two years later WORONIN (**8, 9**) discovered apogamy in *P. tenera* and *P. flavens*. In 1910 the writer reported (**4**) apogamy in *P. atropurpurea* (L.) Link, and later in *P. atropurpurea* var. *cristata* (**7**), *P. viridis* (Forsk.) Prantl (**7**), and in *P. adantoidis* J. Sm. (**6**). PICKETT (**3**) reported apogamy in *P. glabella*. The gametophyte of *Pellaea cordata*, in




FIGS. 1–6.—*Pteris flabellata:* Fig. 1, prothallium from which an apogamous embryo has been produced; ×20. Fig. 2, dorsal view of portion of prothallium showing apical region and origin of embryo; ×100. Fig. 3, portion of prothallium showing apical region before embryo begins development; ×21.5. Fig. 4, dorsal view of apical region of prothallium showing vascular strand extending to apical notch; ×31. Fig. 5, prothallium showing beginning of leaf of embryo; ×20. Fig. 6, ventral view of prothallium showing that the leaf of the young sporophyte has developed far in advance of root; ×20.

which apogamy was also discovered by the writer, becomes heart-shaped and grows to a large size. The wings of the prothallium have a tendency to become lobed even when light conditions appear to be favorable for the growth of the prothallia.

Antheridia but no archegonia have been observed on the numerous prothallia examined.

From studies so far made, the development of the apogamous embryo of *Pellaea cordata* is similar to that of *Pteris flabellata*.

### Apogamy in Cheilanthes feei Moore

So far as the writer is aware, no case of apogamy has heretofore been reported in a *Cheilanthes* species. The gametophyte is considerably smaller than that of either *Pteris flabellata* or *Pellaea cordata*. When the prothallia are grown under the same cultural conditions as those of the other apogamous ferns, they frequently become much branched.

Antheridia are produced in large numbers, but archegonia are never formed.

No unusual features were observed in the development of the apogamous embryo. Occasionally, however, two embryos of apogamous origin were observed to develop from a single gametophyte of *Cheilanthes feei*.

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# MÄULE LIGNIN TEST ON PODOCARPUS WOOD

E. C. Crocker

In 1849, Payen (5) noted that when hemp fibers were treated with chlorine water and then with ammonia they became bright red. This is apparently the earliest record of such a reaction, which was advanced as a lignin test by Mäule (2) in 1901. Schorger (7) noted that the wood of angiosperms gave strong red color with the Mäule test, whereas that of gymnosperms gave only an indefinite brownish gray. The writer (1) confirmed Schorger's observation, and found that the wood of 18 species of angiosperms gave uniformly red coloration, and the wood of 18 species of conifers, and *Ginkgo*, gave indefinite yellow or pale brown color. This distinction has since been further confirmed and extended by Sharma (8) and Schindler (6), and the Mäule test has been more or less accepted as a reliable means of distinguishing between the woods of angiosperms and those of gymnosperms.

The writer has recently used the Mäule test on a great variety of woody fibers from various botanical groups of plants, with interesting results. Many of the rarer of the samples tested were obtained through the kindness of Professor I. W. Bailey, at the Bussey Institution of Harvard University.

The wood from the many angiosperms tested showed, without exception, strong red color when given the Mäule test: treatment of shavings with 1 per cent potassium permanganate, followed by washing, treatment with half-strength hydrochloric acid, washing again, and finally treatment with ammonia. The angiosperms tested included the primitive *Trochodendron*, *Drimys*, and *Tetracentron;* also *Monotropa*, and many other herbaceous plants, including members of the Compositae, Labiatae, Ranunculaceae, and Solanaceae. Material was used also from such woody monocotyledons as various bamboos, palms, *Yucca*, and *Ruscus*.

Thorns of members of the Rosaceae, of *Gleditsia*, and *Berberis* failed entirely to give red color, although in the case of *Gleditsia* there was sufficient lignification to give a red color with phloroglucinol reagent. A section of this wood, with a large thorn growing

from it, was given the MÄULE test. The wood colored deep red, but the thorn and its base within the wood showed the yellow-brown negative test only.

It was found that all specimens of the Gnetales gave strong red coloration. Those tested included tracheids from the roots of *Welwitschia mirabilis*, xylem from the stems of *Ephedra gerardiana*, *Gnetum scandens*, *G. vinosum*, and *G. gnemon*. SCHINDLER (**6**) also secured similar results with the Gnetales.

Xylem from *Lycopodium*, *Aspidium*, *Osmunda*, *Pteris*, *Cycas rumphii*, *C. circinalis*, *Dioon edule*, *Encephalartos hildebrandtii*, *E. villosus*, *E. horridus*, and *Zamia media* gave only the pale brown negative MÄULE reaction, as was also true of the following gymnosperms:

| | |
|---|---|
| Abies balsamea | Libocedrus |
| Agathis robusta | Phyllocladus hypophyllus |
| Araucaria bidwillii | Picea rubens |
| A. cunninghami | P. engelmanii |
| A. araucana | Pinus strobus |
| Callitris calcarata | P. palustris |
| Cedrus sp. | P. taeda |
| Cephalotaxus drupacea | Pseudotsuga taxifolia |
| Chamaecyparis thyoides | Sciadopitys verticillata |
| Cryptomeria japonica | Sequoia gigantea |
| Cupressus macrocarpa | Taiwania sp. |
| Dacrydium cupressinum | Taxodium distichum (?) |
| D. franklini | Taxus sp. |
| Juniperus virginiana | Thuja occidentalis |
| Keteleeria sp. | Torreya nucifera |
| Larix laricina | Tsuga canadensis |

In testing many varieties of coniferous woods, a sample of *Podocarpus pedunculatus*[1] was incidentally encountered which seemed to give a faint reddish coloration, superposed on brown; whereas all other coniferous woods tested up to that time had given only weak brownish color. Numerous repetitions of the test indicated that it was weak but definitely positive.

[1] PILGER (in ENGLER'S Pflanzenreich, 1903) gives *amarus* as the accepted species name, and *pedunculatus* as an unaccepted name for the same species. SWAIN (**9**) used the two names interchangeably.

Through the courtesy of Professor SAMUEL J. RECORD of Yale University, and of Professor H. P. BROWN of Syracuse University, many other samples of *Podocarpus* wood were obtained, all of which (including a specimen of *P. pedunculatus*) gave the usual negative test, excepting two samples of *P. amarus* (from different sources) which gave respectively strong and weak positive tests. Of four samples of this species of wood, from different sources, two gave distinct positive tests, one a weak positive test, and one a completely negative test.

Table I lists the species of *Podocarpus* always giving negative tests.

TABLE I

PODOCARPUS SPECIES GIVING NEGATIVE TESTS

| SPECIES NAME (PILGER SYSTEM)* | NAME ON SPECIMENS AS RECEIVED | SPECIMENS EXAMINED |
|---|---|---|
| coriaceus | coriaceus | 3 |
| dacrydioides | dacrydioides | 6 |
| elatus | elata | 1 |
| elongatus | elongata | 1 |
| ferrugineus | ferruginea | 3 |
| gracilior | gracilior | 1 |
| guatemalensis | guatemalensis | 2 |
| imbricatus | imbricata (2) and cupressina (1) | 3 |
| latifolius | latifolia and thunbergii | 3 |
| ? | lotora | 2 |
| macrophyllus | macrophylla | 1 |
| montanus | taxifolia | 1 |
| nagi | nagi | 1 |
| ? | nakaii | 1 |
| neriifolius | neriifolia (3) and bracteata (2) | 5 |
| novae-caledoniae | novae-caledoniae | 2 |
| ? | philippinensis | 2 |
| salignus | chiliana | 2 |
| spicatus | spicata | 5 |
| totara | totara (8) and hallii (1) | 9 |

* The writer is greatly indebted to Dr. ALFRED REHDER, Curator of the Arnold Arboretum, for checking and comparing species names.

The chemical nature of the material which gives the definite red color in the MÄULE test is still unknown. No estimate has been made of the amount which is present, nor of its possible relation to the material that turns brown in the MÄULE test, which is present in the xylem of conifers, ferns, cycads, and the thorns of *Gleditsia* and

some other angiosperms. It is like phenolphthalein in that its alkaline salt is red-colored and its acid form is colorless.

Some morphologists have surmised that the Gnetales sprang from the Coniferales within or near the *Podocarpus* group. MEZ (3) and his associates, by serological reactions, have come to the same conclusion (4). The findings of the MÄULE lignin test also are consistent with the idea of a possible evolution of the Gnetales from the Coniferales. Further, a particular species (*Podocarpus amarus*) is indicated as the one most nearly like the Gnetales (also the angiosperms, as a group) in exhibiting a particular chemical reaction.

## Summary

1. The MÄULE lignin test is apparently a reliable practical test for distinguishing the woods of the conifers from those of the angiosperms and the Gnetales. The only exception found was the single coniferous species *Podocarpus amarus*.

2. Xylem from the three types of Gnetales, *Welwitschia*, *Gnetum*, and *Ephedra*, all give positive MÄULE tests.

℅ ARTHUR D. LITTLE, INC.
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# BRIEFER ARTICLES

## A NEW METHOD OF DETECTING PATTERNS IN THE RAYS OF FLOWERS

COCKERELL[1] and others have commented on the existence of patterns in the yellow rays of the common sunflower which cannot be detected by ordinary means, the patterns being obscured by the predominance of the yellow pigment. Mrs. COCKERELL discovered a mutant in which this pattern, owing to the unusual development of anthocyanin pigment in the rays, was visible under ordinary conditions. Offspring from this mutant have been cultivated for many years in America, Europe, South Africa, Australia, etc. It was shown by COCKERELL that the pattern in the rays of the ordinary sunflower could be detected in photographs made under certain conditions, even though it could not be seen by direct observation. He suggested that the writer investigate this unusual effect.

It seemed possible that, under certain conditions of illumination, the hidden pattern might be seen directly. In order to test this hypothesis a large Mazda lamp was placed in a projecting lantern mounted in a dark room. A glass water-cell was placed immediately in front of the lantern to remove the infra-red radiation. In front of this was placed at different times many different Wratten Laboratory light filters. These filters, 2 inches square, and made by the Eastman Kodak Company, transmitted only particular wave lengths or particular combinations of wave lengths. If such filters are not available, substitutes can be made by fixing undeveloped photographic plates and staining them with various dyes.

Blossoms of the ordinary sunflower showing no visible pattern in the rays when examined in daylight were examined successively under illumination transmitted by some 60 different filters. When they were examined in the light transmitted by Wratten filter no. 47 the hidden pattern was markedly visible. The rays were green at the tip and red at the base. All of the sunflowers examined showed the pattern although the relative areas of red and green varied with different specimens. Wratten filter no. 47 transmits much of the blue light and a small percentage of the red and green. It transmits practically no yellow, so that the otherwise overwhelming yellow pigment could not obscure patterns which may exist in

[1] Jour. Heredity **6**:542. 1915.

other colors. It transmits, however, sufficient of the red and green light to make patterns in these colors discernible. The pattern was not visible in the light from a filter which transmitted only the blue.

The rays of the common sunflowers while illuminated by light transmitted by filter no. 47 were viewed through a glass prism, which separated the images of the red and green colorations. It was then seen that the intensity of the red coloration faded toward the tips of the rays and that of the green increased. Examination of the rays in this manner when illuminated by white light did not show anything satisfactorily because of the overlapping of the colored images, a condition which does not exist to the same degree in selectively filtered light.

This method of detecting patterns may open a fertile field of research and may lead to the development of visible patterns from those which now are hidden and unknown.—JULIAN M. BLAIR, *Department of Physics, University of Colorado, Boulder, Colo.*

# CURRENT LITERATURE

### Another botanical text-book

Five years of experience with their own classes, and the criticisms, suggestions, and advice from other teachers, have enabled the authors of this widely used text[1] to make considerable change in this edition, while still retaining the general organization and purpose of the book. Some figures have been redrawn and some new ones have been added. The text has been increased, but mostly in the interest of clarity.

It would be interesting in cases like figure 161 to know upon what authority the legend is based. It is doubtless correct, but this stage has been observed so infrequently that botanists would appreciate a reference to the features indicated in the legend. Reconstructions are valuable, pedagogically, but are often inaccurate, as in figures 207 and 222.

The treatment of alternation of generations would seem to give the student a false impression. Plants below the level of sexuality have no alternation of generations. In all plants which have a fusion of gametes, there is an alternation of $x$ and $2x$ phases in the life history. Naturally, at the beginning, as in *Spirogyra*, there is a quick return to the original $x$ condition, and, unless one stops to think, he may not realize that there is any $2x$ phase; but when several cell divisions intervene before return to the $x$ condition, an easily recognizable body with the $2x$ number of chromosomes is built up. The assumption that $x$ and $2x$ genera-

[1] HOLMAN, R. M., and ROBBINS, W. W., Elements of botany. 2d ed. 8vo. pp. viii+404. figs. 268. John Wiley & Sons. New York. 1933.

tions are synonymous with gametophyte and sporophyte generations is responsible for some of the confusion. In such a widely used text, a presentation of the evolution of the $2x$ phase and of the reduction of the $x$ phase in the life history would put more life into the finer morphological part of a book which is so satisfactory in its physiology and in its grosser morphology.

The binding is exceptionally good, since it is not only strong and artistic, but is waterproof and germ-proof.—C. J. CHAMBERLAIN.

### Commercial timbers of India

While intended primarily for those interested in the economic utilization of Indian woods, these volumes[2] have a usefulness that will extend to other groups of scientists. The plant geographer will find the distribution maps which are given for each species a most useful and accurate source of information. These maps would provide material for a detailed study of the forest geography of British India.

For each tree species there is given its complete synonymy, including the vernacular names current in various parts of India, and an extensive bibliography which includes literature on the taxonomy, ecology, physiology, and morphology of the species. The wood is described as to its general characteristics, its microscopic structure, its mechanical properties, its working qualities, and its uses, present and prospective. This description is accompanied by photomicrographs. The habit, size, and distribution of each species are given in detail and the distribution is plotted on a base map which includes British India, Burma, and Siam. In this way not less than 320 species are described and illustrated.

An appendix contains a classification of timbers according to use, a glossary of scientific terms and phrases, and a bibliography. Three indexes give access to the material under trade, vernacular, and scientific names, respectively.

Such a collection of information, coming from authors of undoubted qualification, will make the work a classic that will doubtless be the best source of information regarding the forests and forest resources of India for some time to come.

The printing, illustrations, and binding all seem to have been well done, making a volume that will be indispensable to foresters and plant geographers.—G. D. FULLER.

### The Gastromycetes in Die Natürlichen Pflanzen-Familien

Volume VIIA of the second edition of Die Natürlichen Pflanzen-Familien[3] has appeared. It is devoted to the Gastromycetes and is a contribution by FISCHER. It is characterized by thoroughgoing workmanship and mature judg-

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[2] PEARSON, R. S., and BROWN, H. P., Commercial timbers of India. Vols. I, II. 8vo. pp. 1150. pls. 636. Government of India, Central Publication Branch, Calcutta. 1932. £5.

[3] FISCHER, E. D., Eubasidii: Gastromyceteae. Vol. VII A in Die Natürlichen Pflanzen-Familien (ENGLER) Harms. pp. 122. figs. 91. W. Englemann. Leipzig. 1933.

ment. The volume is printed on good paper; the illustrations are numerous, well chosen, and well done. It is gratifying that this book brings the second edition of the Natürlichen Pflanzen-Familien one step nearer its goal.—G. K. K. LINK.

### Systematic botany

It is gratifying to botanists that the last contribution of the late Professor R. WETTSTEIN is being completed by his son. His is a difficult task: to impart essentially the basic ideas of the creator and yet to mould them so as vitally to incorporate the results of new developments in the field of systematic botany. The first volume of the fourth edition of WETTSTEIN'S Handbuch der Systematischen Botanik[4] has been made available. The first 159 pages are the last completed contribution of the deceased scholar. The high level of achievement of earlier editions is maintained if not excelled. Fossil plants are considered in detail. The illustrations are plentiful and excellent. The volume is a fine example of botanical book-making.

Part I consists of an interesting and instructive contribution to the basic concepts and propositions of phylogenetic botany. Phylogeny and the evolutionary theory are integrated. In the United States there has been a tendency of late to present these separately, with the result that so-called evolution courses are offered in botanical curricula while phylogeny is languishing.

There is an interesting discussion of the significance of alternation of generations in the phylogenetic development of the plant kingdom. The special part of this volume extends from Schizophyta to the class Gnetinae of the Cormophyta.

It is hoped that time will be found for speedy completion of the concluding volumes of this significant contribution to botanical literature.—G. K. K. LINK.

### Tropical woods

Since the previous note[5] regarding this journal, several interesting numbers have appeared.[6] A few of the numerous original articles included in these recent issues are as follows:

STANDLEY, P. C., and STEVENSON, N. S., Two articles on the Cohune palm.

ESPINA, RAMON, and GIACOMETTO, JUAN, Trees of the Sierra Nevada de Santa Marta.

RIMBACH, AUGUST, Forests of Ecuador.

DUCKE, ADOLPHO, Fifteen new forest trees of the Brazilian Amazon.

RECORD, SAMUEL J., Notes on new species of Brazilian woods.

WILLIAMS, L., Peruvian mahogany.

RECORD, SAMUEL J., Notes on tropical timbers.

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[4] WETTSTEIN, R., Handbuch der Systematischen Botanik. pp. iv+537. figs. 555; frontispiece. Franz Deutticke. Leipzig and Vienna. 1933.

[5] BOT. GAZ. 94:429. 1932.

[6] Tropical woods, edited by SAMUEL J. RECORD, School of Forestry, Yale University. 1932, 1933.

STANDLEY, P. C., Three new trees from COLOMBIA.
RECORD, SAMUEL J., The woods of Rhabdodendron and Duckeodendron.
MCLAUGHLIN, R. P., Systematic anatomy of the woods of the Magnoliales.
STANDLEY, P. C., New names for tropical American trees.—A. C. NOÉ.

### Life history of flowering plants of central Europe

A previous note[7] reviewed numbers 31 to 36 of a handbook giving the ecological life history of the flowering plants of Germany, Austria, and Switzerland. Since that time eight more serial numbers (Lieferungen) have appeared.[8] They contain the Dioscoreaceae by W. BRENNER, Ulmaceae by H. WALTER, Amaryllidaceae and Iridaceae by F. BUXBAUM, Orchidaceae by H. ZIEGENSPECK, and Moraceae by H. WALTER. The handbook combines in a very successful manner the ecological, morphological, and taxonomic aspects of the plant families, which are treated in the form of rather brief monographs by the individual collaborators. Ample illustrations are supplied.—A. C. NOÉ.

### Pleistocene paleobotany

In recent years the study of lake bottoms and swamps by means of pollen analysis has made much progress and has greatly increased our knowledge of glacial and post-glacial plant distribution. A very excellent sample of this kind of investigation[9] combines botanical, geological, and anthropological facts in order to attain a clear conception of the changes in the flora of the region around the Federsee (State of Würtemberg, Germany). Climatic changes and the extinction of former plant inhabitants are discussed, as well as the survival of certain glacial types.—A. C. NOÉ.

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[7] BOT. GAZ. 91:222. 1931.

[8] Lebensgeschichte der Blütenpflanzen Mitteleuropas. Edited by WANGERIN, W., and SCHRÖTER, C., Eugen Ulmer, Stuttgart. 1931–1933.

[9] Paläobotanische Monographie des Federseerieds by KARL BERTSCH. Bibliotheca Botanica, No. 103. E. Schweizerbart'sche Verlagsbuchhandlung, 8vo. pp. viii+126. Stuttgart. 1931. Illustrated.